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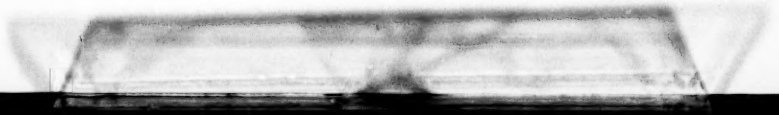

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SEXUAL DIMORPHISM OF SUBMAXILLARY GLANDS IN MICE IN RELATION TO REPRODUCTIVE MATURITY AND SEX HORMONES[1]

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WITH the independent investigations of Lacassagne (1940*a*) and Fekete (1941), the submaxillary glands of mice and rats were established as sex-dimorphic organs. F. S. Hammett (1923) first reported a sex difference in submaxillary gland weight in rats, and Lacassagne later showed that this gland responds in weight and histological appearance to the presence or absence of certain internal secretions.

Subsequent to these initial reports, investigators have described gross, histological, and biochemical responses of the submaxillary gland to extirpation of endocrine organs and to the injection of various hormones, related steroids, and chemical agents.

Endocrine-like activities of the submaxillary gland have been postulated for rats by Higashijo (1940) and for man by Zweifel (1945). The necessity of a functional submaxillary and sublingual gland for survival in 1–10-day-old rats was reported by Plagge (1938).

This paper is a partial report of studies made on the submaxillary gland in mice in which the sex dimorphism of the organ was of chief interest. The following aspects have been studied: (*a*) differences in weight, tubule diameter, and histology of the normal adult male and female submaxillary; (*b*) the time and manner of the onset of sex differences in relation to growth and reproductive maturity; and (*c*) the effects of various sex hormones on the submaxillary gland. The effects of certain mechanical manipulations and secretory drugs on the character of the gland and the comparative histology of the gland among different mammals have also been studied and will be reported in detail in another paper.

[1] The writer is greatly indebted to Dr. Carl R. Moore, who suggested this problem and under whose supervision it was carried out; to Dr. Dorothy Price, who gave helpful criticism; and to John M. Allen for technical assistance. This investigation has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. It is a pleasure to acknowledge the courtesy of Dr. Edward Henderson and the Schering Corporation for supplying androgens (testosterone propionate) and estrogens (estradiol benzoate) and to Dr. M. H. Kuizenka and the Upjohn Company for equine gonadotrophin (Gonadogen).

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MATERIAL AND METHODS

Tri-hybrid mice of Jackson Laboratory origin, colony-inbred in the Hull Zoölogical Laboratory for 5 years, were used throughout the experiments. Between five and six hundred animals were utilized.

At autopsy animals were killed by bleeding, to insure uniform vascular conditions. Organs were dissected with the aid of a wide-field dissecting microscope, and were weighed immediately on a torsion balance accurate to 0.2 mg.

Several fixatives were tested to determine the one best suited for the preservation of histological details to be studied; modified Zenker-formol (Bensley) was the fixative of choice. Iron haematoxylin (Bensley) and Mallory's Triple Connective Tissue Stain were selected for most of the work reported here.

Operative technique, dosages, and methods of administration of hormones will be described with the appropriate experiments.

NORMAL ADULT SEX-DIMORPHIC CHARACTERISTICS

In order to determine normal weights and histology of the mouse submaxillary, both glands from 32 normal males and 18 normal females between the ages of 144 and 175 days were removed, weighed, fixed, and sectioned at 6 μ.

Analysis of the weights revealed a mean weight for males of 114.2 mg. (mean body weight, 27.0 gm.) in contrast to a mean submaxillary weight for females of 67.6 mg. (mean body weight 25.2 mg.). In relation to body weight, therefore, male submaxillaries were 422.9 mg. per cent, whereas those of the female were 268.2 mg. per cent.

Submaxillary glands in mice are compound, branched, tubulo-alveolar glands. A main duct serves all the several lobes of an individual gland as an excurrent opening. Into this duct enter the interlobular connecting ducts, which are continuations of the smaller, branching duct systems of the individual lobules. At the terminations of the duct system are the sexually dimorphic terminal tubules. The acini, which, with the terminal tubules, comprise the morphological secretory unit of the submaxillary, are connected to the tubules by means of short intercalated ducts.

The terminal tubules are the sex-dimorphic constituents of the gland. There is dissimilarity between the terminal tubules of the peripheral or cortical area of the gland and those of the central region. Although some adult male glands may be found in which this difference is not noted, it deserves mention, since it occurs consistently in all adult female submaxillaries and in the submaxillaries of both males and females before puberty. Because of this observation, the tubules will be described as "central" and "peripheral."

In the male the peripheral terminal tubule cell cytoplasm is crowded with acidophilic granules (see Pl. I, Fig. 1), which frequently conceal the basally located nucleus. In the central region the terminal tubule cells contain many small basophilic granules. Some cells are possessed of chromophobe granules, while still others may have a few acidophilic granules among the basophiles.

The diameter of terminal tubules was ascertained by measuring 10 tubules in median sagittal or cross-section from each of 10 different males. The mean diameter was found to be 50.87 μ. The diameter is the same in peripheral and central tubules.

In the peripheral tubule cells of the female, submaxillary acidophilic granules appear characteristically (see Pl. I, Fig. 2) but are very sparse in comparison to

those of the male. The height of the tubule cell is reduced. Nuclei are central, and basal striations appear between the nucleus and the base of the cell except in those cells which contain granules, where the nuclei are basally located and striations tend to disappear. Female tubule cells frequently exhibit a clear or chromophobic area in the apical cytoplasm. This is filled with a fine fibrillar meshwork or chromophobe granules. Such an area is observed less frequently in males and probably is a stage in granule formation.

Central tubule cells in the female have centrally located nuclei, basal striations, and cytoplasm with little granulation.

Measurements of ten terminal tubules from each of ten different females revealed a mean diameter of 31.42 μ. The ranges in tubule diameter in males and females did not overlap.

Acini show no histological differences between males and females. The acini consist of groups of three to six cells organized with their apices together. They possess tiny lumina or none at all. The cell is frequently vacuolated, always basophilic, and sometimes contains fine granules of strongly basophilic material. The nucleus is usually acidophilic, opaque, and basal.

In summary, the submaxillary glands of males, in comparison to those of females, have a greater mean weight and a larger per cent body weight. The terminal tubules are more convoluted, larger in size because of increased cytoplasmic height, and contain greater numbers of granules.

NORMAL GLAND GROWTH AND THE ONSET OF SEXUAL DIMORPHISM

The marked sex difference existing between the submaxillary glands of mature males and females does not exist in young (15–20-day) animals. A chronological study of the growth of the gland was made to determine the age and manner of the onset of the dimorphism in relation to reproductive maturity.

A total of ninety-eight males and ninety-five females from dated litters was used. Autopsy was at 1, 5, 10, 20, 30, 35, 40, 45, and 60 days of age. In addition, three males and three females were autopsied at 25 days of age for histological comparison but are not included in the weight data. At least ten males and ten females are included in each age group, with the exception of day 1, when there are but six of each sex.

For each sex and each age group, data are given in Table 1 on body weight, submaxillary gland weight, milligrams per cent body weight, and tubule diameter. Table 2 provides mean weights of gonads, adrenals, and reproductive accessories from day 20 through day 60. Therefore, the following analysis of the results of periodic study will be confined to histological descriptions of critical stages in the development of sex dimorphism.

DAY 1 THROUGH DAY 20

During this period the submaxillary gland is undergoing developmental changes, and no sex-dimorphic characteristics appear. At birth the gland is made up of tubules and ducts which are indistinguishable from each other, and the acinar system is represented by cells budded from the duct system. Budding increases through 10 days and then decreases slowly. It probably continues for a longer period of time but becomes progressively more difficult to discern as the gland increases in complexity.

The lobules are separated by loose connective tissue, which is the forerunner of the denser septa of the adult gland.

By day 10 configurations of cells are present, which, for lack of previously existent terminology, will be referred to

as "evolving tubules." These are small tubules with basophilic cytoplasmic granules, which, by virtue of their appearance, staining capacity, relation to the duct system, and subsequent response to hormone injections, may be identified as developing terminal tubules. They seem to be equal in number in males and females at the time of their initial appearance.

The sharp increase in per cent body weight noted in Table 1 at 20 days is not to be construed as an indication of sex dimorphism or of maturity. It coincides with the change in food habits which begins at about this age.

DAY 25

Acidophilic granules are first present at this age in the peripheral terminal tubules of both male and female submaxillary glands. This is the initial expression of one of the sex-dimorphic characteristics distinctive in the adult male. There is variation between animals in the number of granules but no sex difference.

A study of the histological condition of the gonads at this crucial age in sub-

TABLE 1

MEAN BODY WEIGHT, SUBMAXILLARY GLAND WEIGHT, MILLIGRAMS PER CENT BODY WEIGHT, AND TUBULE DIAMETER FROM DAY 1 THROUGH DAY 60

Age (Days)	Body Weight (Gm.)		Submaxillary Weight (Mg.)		Mg. Per Cent Body Weight		Tubule Diameter (μ)	
	♂	♀	♂	♀	♂	♀	♂	♀
1	1.4	1.4	6.0± 1.2	5.3±0.8	430	393	.....	.....
5	3.4	3.4	9.2± 2.8	9.7±2.3	265	279	.....	.....
10	6.0	6.0	16.9± 2.2	16.1±2.7	280	248	.....	.....
20	8.0	7.0	36.3± 3.1	33.7±6.7	455	472	.....	.....
30	11.0	11.9	41.3± 9.2	41.3±6.7	372	347	31.16	26.03
35	13.0	12.2	43.0± 6.7	41.4±9.8	330	341	30.54	27.97
40	13.6	12.4	46.2± 9.3	36.5±4.2	336	294	36.96	25.50
45	14.0	14.1	42.5± 7.7	43.8±5.8	302	309	31.68	29.37
60	19.8	16.9	94.5±14.8	61.0±7.3	482	294	48.18	30.47

TABLE 2

MEAN WEIGHTS OF GONADS, ADRENALS, AND ACCESSORY REPRODUCTIVE ORGANS FROM DAY 20 THROUGH DAY 60

Age (Days)	Gonads (Mg.)		Adrenals (Mg.)		S.V. and C.G.* (Mg.)	Uterus (Mg.)
	♂	♀	♂	♀		
20	45.3	2.64	1.86	1.82	1.76	6.7
30	71.2	3.28	2.00	2.03	2.20	7.4
35	91.6	2.35	2.50	2.77	6.87	9.5
40	108.7	1.94	1.82	2.71	7.62	10.4
45	90.0	1.80	2.10	2.88	10.88	10.6
60	152.5	5.70	2.35	3.88	96.12	46.0

* S.V. = seminal vesicle; C.G. = coagulating gland.

maxillary dimorphism brought out that secondary spermatocytes are now present in the testis; the ovary contains many follicles with small antra and well-developed strata granulosa. The X-zone is present in the adrenals of both sexes.

DAY 30 THROUGH DAY 45

Throughout this period there is no abrupt change in the submaxillary gland. In both males and females there is a gradual increase in acidophilic granulation; the intensity of granulation and the number of terminal tubules with granules are greater in some males than in any females, although there is great variation from one animal to another. Weights and tubule diameter measurements show parallel increase in both sexes.

At the beginning of this time interval sperm heads appear in the testes; by 45 days, spermatozoa may be found free in the lumina of the seminiferous tubules of some animals. Ovarian follicles gradually increase in size, but no corpora lutea are found.

At 40 days there appears to be a diminution in amount of granulation and tubule diameter in the submaxillary in the male, which is concomitant with a decrease in actual and submaxillary weight relative to body weight. There is no immediately apparent explanation for this observation, although the reduction coincides with the disappearance of the X-zone in the male adrenal. The X-zone persists in females, and the adrenals, from 40 days on, weigh consistently more than those of the male.

DAY 60

By this age in this strain of mice, some animals are functionally mature, as evinced by the discovery of gravid females at autopsy.

In the male, acidophilic granules tend to permeate central region terminal tubules, and weight and tubule diameter finally become sex-dimorphic.

In the virgin female submaxillary there are the customary few acidophilic granules in the peripheral tubules. The gland of the gravid female contains granules which are equal in quantity and extent to those of any 60-day male.

Spermatozoa are present in some, but not all, testes, indicating that this age is within the period of sexual maturation for males. Weights of the male accessory reproductive organs are within the range of weights for adult males. Follicles and corpora lutea are present in the ovaries, and all stages of the estrous cycle are apparent. The adrenal X-zone is absent in males but present in females.

SUMMARY

Examination of the course of development and establishment of sexual dimorphism of the submaxillary glands in mice in relation to the onset of reproductive development reveals that the two processes exhibit a rough parallelism and that, when sexual maturity is well established at 60 days of age, a marked dimorphism is present in the submaxillary gland.

In each case variability is present among different animals. Thus some males had spermatozoa in seminiferous tubules at 45 days, while they did not occur in other males until after 60 days. Similarly, submaxillary glands do not exhibit dimorphism on the basis of mean weight, milligrams per cent body weight, tubule diameter, and state of granules until the time of reproductive maturity, although it is presaged from 35 days onward.

It is of interest to note that the acidophilic granules appear between 20 and 25 days of age. The initial appearance

is at the same time in both sexes. The granule dimorphism is the first dimorphism to be indicated, and weight and tubule diameter dimorphisms follow later.

GONADECTOMY IN MALES

Granules appear very early in the course of normal development of the gland and occur characteristically in the adult male. Testes were removed from prepuberal and mature males to test the effect of testis-secreted androgen on the assumption and maintenance of normal male tubule granulation and on submaxillary weight.

TABLE 3

MEAN SUBMAXILLARY WEIGHT, MILLIGRAMS PER CENT BODY WEIGHT, AND TUBULE DIAMETER IN ADULT AND PREPUBERALLY CASTRATED MALES

Age at Operation (Days)	Age at Autopsy (Days)	No. of Cases	Submaxillary Weight (Mg.)	Mg. Per Cent Body Weight	Tubule Diameter (μ)
10.....	64	5	58.2	265.5	29.62
105.....	170	4	62.6	223.6	31.35

Four males were bilaterally castrated at the age of 105 days and autopsied at 170 days, after 65 days of castration. Five males were bilaterally castrated at 10 days and autopsied at 64 days, following 54 days of castration.

Table 3 shows mean weights of submaxillaries, milligrams per cent body weight, and mean tubule diameter measurements.

PREPUBERALLY CASTRATE MALES

In histological appearance the submaxillary glands of males castrated at 10 days and autopsied at 64 days are essentially like those of the adult female except that terminal tubule granules are fewer and the tubule diameter is smaller than in the normal adult female (see Pl. I, Fig. 3). Clear areas are present in tubule cells, nuclei are central, and basal striations are prominent. It is noteworthy that granules are still present in this gland, although they are rare and are restricted to the periphery.

ADULT CASTRATED MALES

Although granules are diminished in the tubule cells, they do not completely disappear and remain especially prominent at the periphery of the gland (see Pl. I, Fig. 4). The nuclei of the cells tend to assume a more central position, and basal striations reappear.

Granules, then, do not disappear after long castration in the adult male, nor do they fail to form in males castrated previous to the time of their normal appearance. Thus androgens of testicular origin do not appear to be necessary for a minimal number of granules. Removal of the testes following reproductive maturity and the attainment of normal submaxillary sex dimorphism is followed by loss of gland weight and reduction in percentage body weight and tubule diameter until the submaxillary of the castrate male is comparable to that of the normal virgin female. When the testes are extirpated at 10 days of age, the submaxillary not only fails to attain normal weights and tubule diameter by 64 days but falls short of the mean measurements for normal 60-day females.

GONADECTOMY IN FEMALES

Castration of males indicated that the submaxillary normally responds to testicular androgens by weight, and tubule diameter and granule production increase. However, since the female gland produces granules at the same time as does the male, the possibility remains that

estrogens may act to inhibit the assumption of the characteristics thought of as typically masculine. To test this possibility, females were gonadectomized prepuberally and after reaching reproductive maturity, and their submaxillary glands were subsequently examined for indications of increment in the male direction.

Nine females were bilaterally ovariectomized at 10 or 12 days. Of these, four were autopsied at 72 days, 60 days after spaying, and five were autopsied at 100 days, 90 days after spaying. Five 110-day-old female controls and five 74-day controls were autopsied for comparison.

Twelve females were ovariectomized at 60 days. Five were autopsied 45 days later, at 105 days of age; seven were autopsied 90 days later at 150 days of age. Five normal virgin females were autopsied at 150 days of age for comparison.

Analysis of mean weights of the submaxillaries, milligrams per cent body weight, tubule diameters, adrenal weights, and uterine weights is presented in Table 4.

Regardless of the age at time of operation or autopsy, the histological appearance of the female submaxillary is essentially the same, and the following description applies to all such glands.

Acidophilic granules continue to appear in peripheral terminal tubules, except in females spayed at 12 days and autopsied at 72 days; but there is no indication of their increase following ovariectomy. In fact, the change, if any, is reduction in granule number, both centrally and peripherally. There are no other changes in tubule cell characteristics.

Acinar cell size, on the other hand, is notably reduced following ovariectomy (see Pl. I, Fig. 6). The size of individual cells is reduced, the staining capacity is slightly modified, and the cells lack the granular appearance of normal acinar cells.

Examination of control female submaxillaries shows that there are, in general, a few more tubule cell granules present than in the glands of spayed females and that acini are larger.

Ovariectomy, therefore, tends to reduce the weight of the submaxillary and decrease the size of the acini. There is a tendency, correlated with loss of granular inclusions, toward reduction in tubule diameter, although the diminution is not universal. In the times employed, there was no suggestion of masculinization of the female submaxillary gland in the absence of ovarian hormones.

TABLE 4

MEAN WEIGHTS OF SUBMAXILLARY GLANDS, ADRENALS, AND UTERI, MILLIGRAMS PER CENT BODY WEIGHT, AND TUBULE DIAMETER FOLLOWING OVARIECTOMY

Age at Operation (Days)	Age at Autopsy (Days)	No. of Cases	Submaxillary Weight (Mg.)	Mg. Per Cent Body Weight	Uterus (Mg.)	Adrenal (Mg.)	Tubule Diameter (μ)
10	100	4	58.7	315	9.6	3.5	29.93
12	72	5	57.0	306	6.4	3.6	27.32
60	105	5	55.4	270	8.5	3.9	29.00
60	150	7	56.5	265	7.8	4.0	31.25
Control ♀ ♀	110	5	67.3	337	123.3	4.5	33.11
Control ♀ ♀	150	5	63.5	283	60.3	4.5	31.63
Control ♂ ♂	74	5	110.1	470	.....	3.3	49.34

EFFECTS OF ANDROGENS

If granule formation and weight differences in submaxillary sex dimorphism depend, for their fullest expression, on the presence of testicular androgens, injection of exogenous androgens should result in precocious establishment of masculine characteristics with equal facility in immature males and females. To test this hypothesis, testosterone propionate was injected into littermate males and females in varying amounts and over different periods from 10 to 30 days of age.

MALES

A total of thirty immature males was employed. Four were injected subcutaneously with 0.1 mg. testosterone propionate daily from day 10 through day 19 and sacrificed the following day. Three more were injected with 0.05 mg. testosterone propionate from day 10 through day 29 and autopsied the following day. Five 15-day control males and seven 30-day control males were sacrificed for comparison.

Table 5 presents organ weights and milligrams per cent body weight for experimental and control males.

Animals that received a total dose of 0.5 mg. of testosterone propionate from day 10 to day 15 showed no masculinization as a result of the treatment; neither precocious terminal tubule granulation nor increase in actual or per cent body weight occurred. That the level of androgen and time of treatment were effective for other target organs is revealed by the seven fold increase in reproductive accessory gland weights of experimental males over control males.

TABLE 5

MEAN SUBMAXILLARY WEIGHT, MILLIGRAMS PER CENT BODY WEIGHT, AND COMBINED SEMINAL VESICLE–COAGULATING GLAND WEIGHT OF IMMATURE MALES INJECTED WITH TESTOSTERONE PROPIONATE

Amount of Daily Injection (Mg.)	Duration of Treatment (Days)	No. of Cases	Submaxillary Weight (Mg.)	Mg. Per Cent Body Weight	Combined S.V.–C.G. Weight (Mg.)
0.1	10–15	4	20.7	345	7.0
Controls		5	20.2	396	0.96
0.1	10–20	6	28.3	382	6.1
Controls		5	34.7	475	1.9
0.05	10–30	3	54.1	470	48.7
Controls		7	36.3	361	2.2

However, there was a marked increase in the number and size of evolving tubules and in the amount of their basophilic granulation. This observation suggested that the lack of response of the 10–15-day submaxillary to injected androgen is due to inadequate stimulation or, more probably, to inability of the gland to respond because competent elements are not yet differentiated.

At 20 days, following a total testosterone propionate administration of 1.0 mg. over 10 days, acidophilic granules are observed in the peripheral terminal tubules, and clear areas, which precede granule formation, appear frequently within tubule cells. The extent of granulation is comparable to that in a normal 30-day male. Evolving tubules again are

more numerous, and the amount of their granulation is increased.

Actual and per cent body weight are not increased at 20 days by androgen administration, although combined seminal vesicle–coagulating gland weights reveal that these organs responded to the dosage administered. Comparison of the weights of 20-day accessory reproductive glands with those from the 15-day injected animals suggests that the response in both instances was maximal.

When a total of 1.0 mg. testosterone propionate was injected from day 10 to day 30, the masculinization of the submaxillary was unmistakable. Tubules were filled with acidophilic granules, and tubule diameter was comparable to that of adult males. Actual and relative body weights show a significant increase over the weights of the controls. Evolving tubules are infrequently observed, and tubule nuclei assume basal positions in the cells.

FEMALES

A total of twenty-seven immature females was employed. Four were injected with 0.1 mg. testosterone propionate daily from day 10 through day 14. Two females received 0.1 mg. testosterone propionate from day 10 through day 19, and four females were given 0.05 mg. testosterone propionate daily from day 10 through day 29. All were sacrificed on the day following the final injection. Five 15-day, five 20-day, and seven 30-day control females were autopsied for comparison. Table 6 contains mean submaxillary weights and milligrams per cent body weight for the injected females and controls.

Examination of the weight data reveals that, as in the males, gross weight and per cent body weight did not increase after androgen injection during days 10–15 or 10–20. In fact, in both actual and relative weights there is a decrease. Injections during days 10–30, however, caused a sharp increase in actual and relative weights, females so treated surpassing littermate males in the latter.

Histological examination reveals that the female gland responds in the same manner and at the same times as does that of the male. That is to say, injections from day 10 to day 15 cause increase in number, extent, and basophilic granulation of the evolving tubules; injections from day 10 to day 20 produce acidophilic granules in peripheral tubules; and injections from day 10 to day 30 cause the immature female gland to appear in all respects, except gross weight, like that of the adult male gland.

TABLE 6

MEAN SUBMAXILLARY WEIGHT AND MILLIGRAMS PER CENT BODY WEIGHT OF IMMATURE FEMALES INJECTED WITH TESTOSTERONE PROPIONATE

Amount of Daily Injection (Mg.)	Duration of Treatment (Days)	No. of Cases	Submaxillary Weight (Mg.)	Mg. Per Cent Body Weight
0.1	10–15	4	20.6	317
Controls		5	23.4	371
0.1	10–20	2	25.3	361
Controls		5	27.9	442
0.05	10–30	4	49.3	513
Controls		7	38.2	336

These two experiments revealed that a distinctive masculinization of the immature submaxillary gland could be attained with androgen stimulation after injections which were maintained to 30 days of age. This masculinization involved increase in acidophilic granulation, enlargement of tubule diameter, and increase in gross and percentage body weight. The gross weight of the female gland is less than that of the immature

male gland at the same age and same stimulation. Precocious granulation could be produced at 20 days but not at 15 days with the amounts of testosterone employed. Androgen injections stimulated the number, size, and basophilic granulation of the evolving tubules, offering additional proof that they are indeed the differentiating terminal tubules.

CASTRATE MALES

Gonadectomy of males, as reported above, produces submaxillary glands that are essentially feminine in appearance and weights; the granulation is sparse and confined to the periphery of the gland. Injections of testosterone propionate were made into castrated males, operated and autopsied simultaneously with castrated uninjected males, to test the rapidity and nature of response to exogenous androgens. Normal virgin females were given the same injections and sacrificed for comparison.

Prepuberal castrated males.—Two males were castrated at 10 days of age, injected subcutaneously with 0.25 mg. testosterone propionate daily from the 59th to the 63d day following castration, and autopsied on the next day.

The mean weight of the submaxillary gland after injection of a total dose of 1.25 mg. testosterone was 91.6 mg., representing a milligram per cent body weight of 366.4. The tubule diameter was 44.26 μ. These figures reveal a considerable increase in comparison with castrate males of the same age and duration of castration (see Table 3) and are comparable with mean measurements of normal 60-day males (see Table 1).

Histological examination shows that cell height in the terminal tubules increased sharply, and clear areas are noticeable in all central tubule cells. Granules are formed peripherally but are very rare in the tubules of the central region. A few basal striations are present.

Adult castrated males.—Four males castrated at 105 days and allowed to remain so for 60 days were injected with 5 daily doses of 0.25 mg. testosterone propionate from day 165 to day 169 and were autopsied at 170 days of age.

The mean gross weight of the submaxillary glands was 113.0 mg., and the milligram per cent body weight was 403.6. Tubule diameter was 47.02 μ. These measurements show a sharp increase over those of the untreated castrate male of the same age and duration of castration, exceed those of the prepuberally castrate male under the same androgenic stimulation, but are less than those of the normal adult male.

Examination of sections reveals that the cytoplasm of the terminal tubule cells is greatly increased in height, clear areas are present, and granules are formed in central terminal tubules. At the periphery the tubule cells are distended with granules and appear completely restored in comparison with granulation in normal male tubules (see Pl. I, Fig. 5).

NORMAL VIRGIN FEMALES

Three 60-day-old virgin females, littermates of the prepuberally castrated males, were injected with five daily doses of 0.25 mg. testosterone propionate and autopsied at 65 days of age, to compare their response to androgen with that of both immature and adult castrated males. The submaxillary weight was 78.1 mg., which represents a milligram per cent body weight of 410.5. Tubule diameter was 46.41 μ.

Clear cytoplasm in the tubule cells is a prominent feature of the sections examined. Granules form in the peripheral terminal tubules in numbers that com-

pare favorably with those of the injected castrated males and also appear in the central tubule cells to some extent. The nuclei of tubule cells are located basally, but basal striations are still present, particularly in cells that do not contain granules.

These three experiments show that injections of androgens into prepuberally castrate males increase gross weight, milligrams per cent body weight, tubule diameter, and peripheral granulation of the submaxillary gland. Similar injections into adult castrated males brings weights, diameter of tubules, and histology nearly to the level of normal males and exceeds the increase noted in the younger males. Littermate females of 65 days of age respond by increases greater than that of their littermate males but less than that of the adult males.

EFFECTS OF ESTROGEN

Since the ovariectomy experiment reported above indicated that lack of ovarian hormone caused the submaxillary gland of the female to suffer reduction in weight, tubule diameter, and acinar size, it was necessary to test the reparative faculties of exogenous estrogens.

Three females were ovariectomized at each of the following ages: 12 days, 10 days, and 60 days and were autopsied at 60 and 90 days following the operation. During the 10 days preceding autopsy, each female received 10 RU daily of estradiol benzoate injected subcutaneously. Five 110-day and five 150-day spayed females from the ovariectomy experiments previously reported were used as controls. Table 7 includes mean organ weights, milligrams per cent body weight, and tubule diameter of experimental and control animals.

It may be seen from Table 7 that the submaxillary weight and milligrams per cent body weight of the injected animals are greater than those of their controls and that tubule diameter is equivalent.

TABLE 7

ORGAN WEIGHTS, MILLIGRAMS PER CENT BODY WEIGHT, AND TUBULE DIAMETER IN FEMALES FOLLOWING ESTROGEN INJECTION

Age at Operation (Days)	Age at Autopsy (Days)	No. of Cases	Amount of Injection (RU)	Submaxillary Weight (Mg.)	Mg. Per Cent Body Weight	Uterus (Mg.)	Adrenal (Mg.)	Tubule Diameter (μ)
12	72	3	100	79.9	395	81.3	5.3	31.27
10	100	3	100	79.0	384	106.2	4.6	31.43
Control	110	5	.....	67.3	337	123.3	4.5	33.11
60	150	3	100	66.3	300	132.8	4.8	35.92
Control	150	5	.....	63.5	283	60.3	4.5	31.63

The histological response of the animals autopsied at 72 days of age and 100 days of age reveals primarily that the acini are markedly larger in size, this being determined by increase in size of individual cells (see Pl. I, Fig. 7). The acini are distended, and granules are very small or absent. Terminal tubule granulation is equivalent to that of the controls.

The females autopsied at 150 days and after estrogen injection have more acidophilic granulation in the peripheral terminal tubules than the normal females, and there is an increase in the number of cells which show clear areas. Acini are much larger in one of the injected females; the other two had acini

comparable to those of females ovariectomized for an equivalent length of time. That is to say, the response to injected estrogen was like that of the 72- and 100-day females in one instance, while there was no response in the other two 150-day estrogen-treated females.

Injection of 100 RU estradiol benzoate during 10 days preceding autopsy in ovariectomized females increased all the weights under consideration to a level exceeding that of the controls. It tended to increase acinar cell size and prevalence of terminal tubule granules. Estrogen injection repaired all the changes caused by ovariectomy.

It is noteworthy that the adrenals increased in weight following estrogen injection. This, coupled with the fact that adrenal weight decreases following ovariectomy, suggests that the changes in submaxillary weight and histology might be mediated by the adrenal and be but secondarily affected by estrogens.

GONADOTROPHINS IN IMMATURE MALES AND FEMALES

Since the effect of exogenous androgen and estrogen on the submaxillary gland had been demonstrated, a final experiment was made to test the response of the submaxillary glands of immature males and females to endogenous gonadal hormones produced by gonadotrophic stimulation.

Five males and four females were injected subcutaneously with an aqueous solution of gonadotrophin amounting to 0.5 Cartland-Nelson unit per day from day 20 to day 34 and were sacrificed on the following day. Three male and four female controls were autopsied for comparison at 35 days.

TABLE 8

MEAN WEIGHTS AND MILLIGRAMS PER CENT BODY WEIGHT OF SUBMAXILLARY GLANDS IN IMMATURE MALES AND FEMALES INJECTED WITH GONADOTROPHIN

	Body Weight (Gm.)	Sub-maxillary Weight (Mg.)	Mg. Per Cent Body Weight	Organ Weight				Tubule Diameter (μ)
				Gonad	V.P.*	C.G.-S.V.†	Ut.‡	
Control males	14.7	49.6	338	105.8	1.1	9.5	.....	41.9
Injected males	14.0	52.9	378	97.0	8.6	102.0	.....	48.9
Control females	12.9	47.3	368	2.1	.....	.....	11.9	33.1
Injected females	14.0	49.7	355	12.2	.....	.....	52.0	33.9

* V.P. = ventral prostate.
† C.G.-S.V. = combined seminal vesicle-coagulating gland weight.
‡ Ut. = uterus.

Following injections, the male gland increased very slightly in actual weight and in milligrams per cent body weight (Table 8), although examination of reproductive accessory weights shows that adequate androgenic stimulation was available.

However, histological examination reveals that tubule diameter is increased to nearly that of the adult male, and in granulation and cellular criteria the gland is comparable to that of the adult male. Evolving tubules appear in the gland as they did after injection with exogenous androgen, but they lack the intense basophilic staining capacity that they possess after androgen injections.

The male control gland at this age has acidophilic granules at the periphery and basophilic and chromophobe granules in central tubules. The gland is, however, still immature and lacks typical masculine characteristics.

No changes in weight and tubule diameter are apparent after gonadotrophin injection in females. The control glands are essentially like those of 35-day females previously described.

The submaxillary glands of 35-day-old males, then, can respond by tubule diameter increase and augmentation of granulation to approach that of normal adult males in response to androgen presumably secreted by the gonadotrophin-stimulated testis. Increase in weight to adult levels does not occur at this age with this dosage of gonadotrophin, although seminal vesicle and coagulating gland weights are within the range of normal adult weights.

The submaxillary glands of young females do not show any effect resulting from injections of gonadotrophin, although ovaries and uteri are greatly enlarged.

DISCUSSION

The components of the sexual difference of the submaxillary glands in mice as reported in this paper are greater gross weight, greater weight in relation to body weight, larger terminal tubule diameter, and greater numbers of granules in the tubule cells of males than females. These observations confirm the reports of earlier workers (Lacassagne, 1940*a*, *b*, *c*; Lacassagne and Chamorro, 1940; Fekete, 1941; Caussé and Lacassagne, 1942; Feyel-Cabanes, 1947; Frantz and Kirschbaum, 1949*a*).

The secretory activity of the tubule cells, which is reflected in granule amount, nuclear position, basal striations, and tubule diameter, appears to be affected markedly by the presence or absence of male hormone and to some extent by female hormone.

My results show that removal of the testes at 10 days, with autopsy at day 65, completely prevents the assumption of all sexually dimorphic characteristics, and the gland is indistinguishable from that of the normal female of the same age except for the fact that the castrated male has even fewer granules in terminal tubule cells than does the normal female.

The results of castration in decreasing weight and tubule diameter and in causing depletion of granules and general feminization of the gland have been reported by Lacassagne (1940*a*, *b*, *c*), Lacassagne and Chamorro (1940), and J. Raynaud (1946). Subsequent work by other authors has confirmed their results, as do the observations reported in this paper.

Exogenous androgen injected into castrate males, normal virgin females, and immature males and females quickly induced typical masculine appearance and evoked weight increase of the submaxillary gland. In general, females did not attain so great a weight as did castrated littermate males when injected with the same amount of androgen over the same period of time. This may indicate that the female gland is not capable of responding so rapidly or so much as the male gland.

On the other hand, females injected with androgens were capable of greater tubule diameter and granule production increase than males castrated previous to the 10th day of life. This might be ascribed to a priming action of estrogenic steroids or adrenal influences which enhance the ability of the female terminal tubule to react to androgens.

The action of androgens upon the submaxillary glands of normal adult males, castrated males, and hypophysectomized males has been reported by Lacassagne (1940*a*, *b*, *c*), Lacassagne and Chamorro (1940), Feyel-Cabanes (1947), and Frantz and Kirschbaum (1949*a*) and in the castrated thyroidectomized male rat by Grad and Leblond (1949).

In the immature male and female (10–30 days) granules could be induced by androgen or gonadotrophin injection only at or near the age when they normally occur. The experiments reported here reveal that granules, which appear normally at 25 days, can be produced precociously at 20 days by androgen injection, but not at 15 days. However, this does not mean that there was no response previous to 20 days; at 15 days precocious stimulation was expressed by increase in the gland components, the evolving tubules, which would later respond by granule formation.

My experiments reveal that gonadectomy in adult females produces a pattern of slight reduction of weight, tubule diameter, and granule production in addition to diminution of acinar size. These changes are subtle but constant during the times involved.

It has been reported by Chaulin-Servinière (1942) and J. Raynaud (1943) that spaying of adult females results in masculinization of the submaxillary gland after several months. This condition, they state, is variable and appears only after prolonged ovarian deprivation. Raynaud (1944) reports that ovariectomy at birth does not result in masculinization of the submaxillary gland.

In a series of female mice gonadectomized at 1–3 days and autopsied at 6–12 months of age, masculinization of the submaxillary gland is reported by Woolley and Little (1945*a*, *b*). Females that showed masculinization also had adrenal tumors, while those that retained the female-type submaxillary either had no adrenal tumors or had aberrant tumors. Masculinization in adult ovariectomized females was also attended by adrenal tumor formation. Analysis of sex accessories in these females showed effects attributable to estrogen and androgenic hormones, presumably secreted by the adrenal neoplasm. Frantz and Kirschbaum (1949*b*), also using the submaxillary of ovariectomized mice as a criterion for the type of hormone secreted by adrenal tumors, reported that submaxillaries were masculinized if other accessories showed evidence of androgen secreted by the tumor and were female if the tumor secreted estrogen.

The experiments reported in this paper showed no masculinization of the submaxillary in either adult or prepuberally ovariectomized females over periods as long as 3 months. Examination of the adrenals from these females showed no signs of adrenal tumor formation.

The injection of estrogens reported here provides further proof of the response of the submaxillary to ovarian hormones, since estradiol benzoate evoked repair of all changes resulting from ovariectomy in both young and adult females. Tubule diameter, weight, granule production, and acinar size were increased.

Lacassagne (1940*b*) reported an increase in tubule diameter in castrate males and females after injections of estradiol benzoate and also (1940*a*) that estrogens caused increase in acinar size.

It is difficult to reconcile the reports of masculinization after ovariectomy with the results of estrogen injection. It is possible that masculinization, when it occurs, is mediated in some way by adrenal hormones, hastened and intensi-

fied by adrenal tumor formation. If estrogens per se affect the submaxillary gland, it is in a much more subtle manner than with androgens, although in the same direction. There is a possibility that adrenal weight decrease following gonadectomy and increase following estrogen injection are related to the submaxillary changes.

It was noted throughout the series of observations and experiments reported here that the peripheral terminal tubule cells are more capable of producing, retaining, or storing granules than are those of the central region. Granules are almost restricted to the periphery in the normal adult female and are more numerous in the periphery than in the central region in the normal adult male. They appear first in the periphery of both males and females at 25 days of age and are retained there following castration. Androgen injection produces granules first in the peripheral tubules, and only later are granules found to have invaded the central tubules. Estrogen injection tends to increase peripheral granulation in females.

This peripheral-central variation has been noted by Feyel-Cabanes (1947*b*), who goes so far as to term it a "cortical-medullary" difference. Junqueira *et al.* (1949) refer to this variation in their reports of histochemical investigations of the mouse submaxillary.

There is no readily available explanation for these observations concerning peripheral sensitivity, and other methods than those used here must be employed to explain it.

It seems well established by orchidectomy, androgen injection, and gonadotrophin injection in immature animals that testicular androgens play an important role in synthesis of granules in the submaxillary gland. However, minimal numbers of granules are produced in young animals, normal females, and castrated males. Therefore, we must assume that the testicular stimulation is superimposed upon a fundamental biological capacity for granule production and/or some nonsexually dimorphic instigation from another source.

It must be noted in relation to the action of hormones upon the submaxillary gland that Feyel-Cabanes (1947*b*) believes cholesterol to be effective in promoting synthesis of granules in the terminal tubule cells of castrated male mice. J. Raynaud (1948) failed to find that cholesterol repaired castration-induced degranulation. Frantz and Kirschbaum (1949*a*) report that adrenotrophins cause granulation in terminal tubules. Thus it appears that the submaxillary gland is sensitive to various steroid molecules, with androgens and closely related adrenal steroids as the most effective of them.

The study of the onset of sexual dimorphism, which was undertaken as a necessary corollary to the understanding of the sex difference in the adult submaxillary gland, has shown the gland to be in a very immature condition at the time of birth. It is made up of ducts and presumably nonsecretory tubules.

The gland lacks definite acinar cell configurations at day 1, but these cells are in the process of being budded off from the tubule-duct system. The staining capacity of the budded cells and the nature of their subsequent development suggest that they are secretory from the time of their appearance. Plagge (1938) has shown that removal of submaxillary glands at birth prevents the mechanical process of attachment of the suckling young to the nipple.

Borghese (1950) states that acini are established by the 17th day of embryonic development in the mouse, but it is not

clear whether he refers to acini or to the herein designated "terminal tubules." He also observed the ramifications of the tubule system by means of branching in normal embryonic development, and my experiments show that this ramification continues after birth and up to at least 30 days of age. M. Heidenhain (1921) reported the extension of tubules by bifurcation and the appearance of acinar cells by budding from the tubules ("adenomeren theory") in human embryo submaxillary glands.

Developing terminal tubules (evolving tubules) may be distinguished in the immature submaxillary gland by virtue of their characteristic cellular appearance and intense basophilic staining. These eventually are converted into terminal tubules in adults but persist longer in females than in males.

The relation of the development of sexual dimorphism of the submaxillary gland to the attainment of sexual maturity, although closely related in time, is not demonstrated as strictly dependent upon the latter physiological process. Reproductive capacity, in the main, is attained by day 60, since, in some cases, brother-sister matings have resulted in pregnancies. By this period, also, the sexual dimorphic elements, such as greater gross and percentage body weight, greater tubule diameter, and more intense granule formation in male terminal tubules, are definitely established. One has not, however, ruled out the influence of progressive body development as a whole or the influence that other organs of internal secretion may have upon the submaxillary gland.

Throughout the extent of these experiments I have attempted to determine whether the submaxillary gland is neuter in the female and whether maleness is a condition bestowed by the male endocrine complex or whether there is some genetic difference between the glands of the two sexes. With the experimental procedures brought to bear upon this question, only gross weight increase of the gland indicates a difference that is sex-linked.

SUMMARY AND CONCLUSIONS

1. The submaxillary glands of adult male and female mice exhibit a sex dimorphism which is characterized by greater gross and percentage body weight, greater terminal tubule diameter, and more intense granule production in tubule cells of males than of females.

2. Examination of histological and weight changes of the submaxillary gland in relation to reproductive maturity from day 1 to day 60 reveals that assumption of sex dimorphism in the submaxillary is roughly parallel to attainment of reproductive maturity. Granules are formed in terminal tubule cells at about 25 days and gradually increase in both sexes, with the male showing a regularly greater increment in their production than the female. Weight and tubule diameter dimorphism are not fully established in this strain of mice until between 45 and 60 days, by which time these animals are reproductively mature.

3. Testosterone propionate injected into 30-day males and females can cause tubule diameter and granule production to increase to a stage that is essentially like that of the normal adult male.

4. Injection of gonadotrophins into 35-day males causes them to assume the male sex-dimorphic characteristics. Injected gonadotrophins have no effect on the 30-day female submaxillary.

5. Terminal tubule granules are the first of the sexually dimorphic elements to appear normally and are more sensitive to hormonal stimulation than other criteria.

6. Removal of testes prepuberally or

following reproductive maturity results in loss of characteristic male appearance, and the gland assumes the appearance of the normal female submaxillary.

7. Androgens injected into adult castrated males, prepuberally castrated males, and normal young adult virgin females causes the assumption of characteristic male appearance and increase in weight of the submaxillary.

8. Ovariectomy is followed by slight reduction in weight, granule production, and tubule diameter and a marked reduction in acinar size.

9. Injection of estradiol benzoate not only repairs the changes caused by ovariectomy but causes a slight increase over the controls in all criteria except tubule diameter in prepuberally ovariectomized females.

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PLATE I

FIG. 1.—Cross-section of submaxillary gland of a normal adult male mouse, showing prevalence of terminal tubules in relation to acini, intensity of acidophilic granulation, and large tubule diameter. Note peripheral-central difference in tubule granulation. Zenker-Mallory, ×95.

FIG. 2.—Normal adult female submaxillary gland, showing prevalence of acinar tissue, small tubule size, and acidophilic granulation in peripheral terminal tubules. Zenker-Mallory, ×95.

FIG. 3.—Submaxillary gland of male castrated at 10 days of age and autopsied at 64 days of age. Note slight granulation, central position of nuclei, and basal striations. Zenker-iron haematoxylin, ×380.

FIG. 4.—Submaxillary gland of male castrated at 105 days of age and autopsied at 170 days of age. Note persistence of granules at periphery. Zenker-iron haematoxylin, ×380.

FIG. 5.—Submaxillary gland of male castrated at 105 days of age and masculinized following injection of a total of 1.25 mg. testosterone propionate during 5 days preceding autopsy at 170 days of age. Zenker-iron haematoxylin, ×380.

FIG. 6.—Submaxillary gland of female castrated at 10 days of age and autopsied at 100 days of age. Acini are small and darkly staining. Few granules appear in terminal tubules. Zenker-Mallory, ×380.

FIG. 7.—Submaxillary of female castrated at 10 days of age and injected with 100 RU estradiol benzoate during 10 days preceding autopsy at 100 days of age. Acinar size is markedly increased, and granule production is intensified. Zenker-Mallory, ×380.

RESPONSES OF THE DISTAL RETINAL PIGMENT OF PALAEMONETES TO ILLUMINATION[1]

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For a number of years investigators working in the field of crustacean retinal pigment physiology have referred to a light-adapted position and a dark-adapted position of the distal retinal pigment. The shift of this pigment from one of these positions to the other is generally considered to constitute a part of the mechanism for regulating the amount of light entering the eye during the day and night, thus producing optimum vision under these two conditions of illumination. The research which has dealt with the mechanics of the retinal pigment movements from one position to the other and factors influencing this change has been amply reviewed by Welsh (1930) and Parker (1932).

The techniques used in most of the investigations were relatively time-consuming and therefore not favorable for the collection of data from a large number of animals. They involved sacrificing the animals, fixing the eyestalks, and determining with an ocular micrometer the position of the retinal pigments. Intermediate stages during the process of pig-

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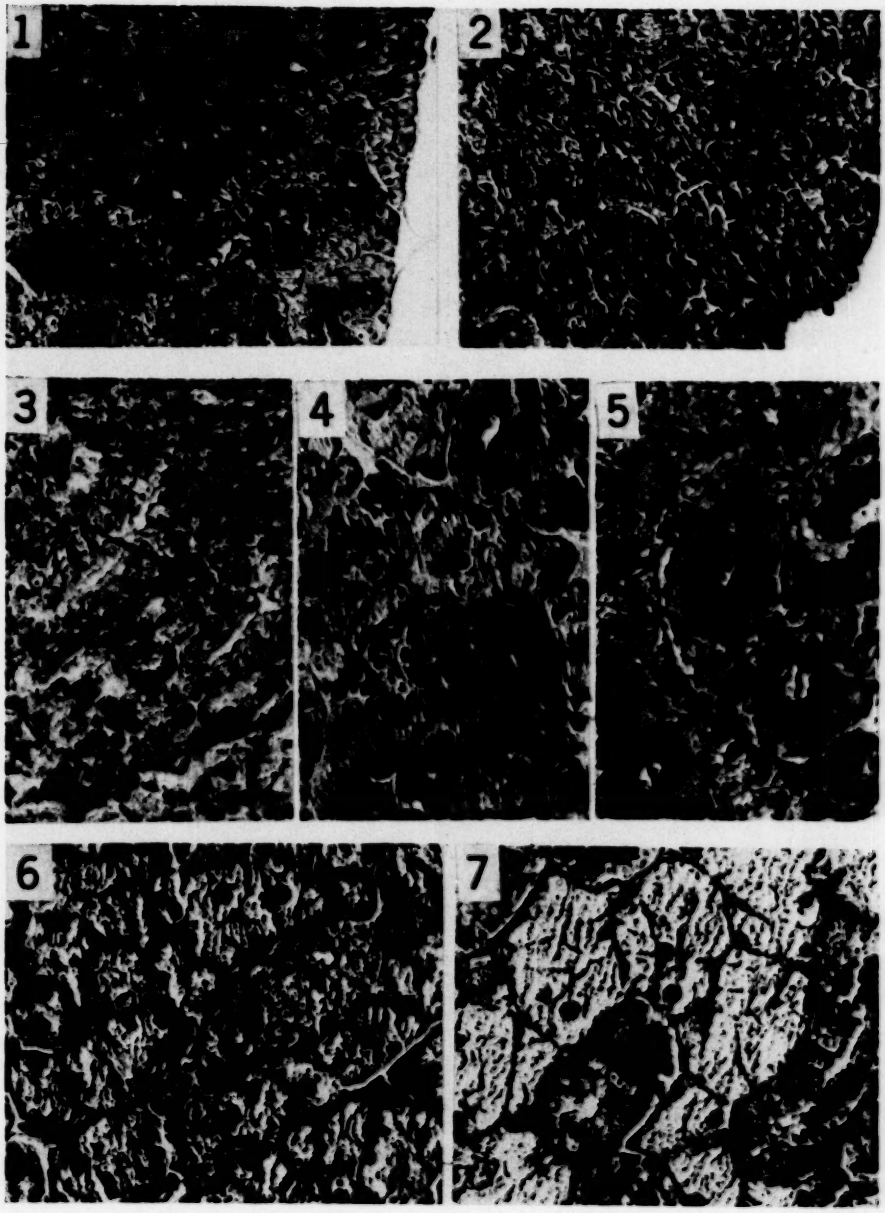
PLATE I



ment migration were obtained by sacrificing animals at various intervals following the transfer from light to darkness or from darkness to light. In this manner Parker (1897) established for the prawn *Palaemonetes* that proximal migration of the distal retinal pigment in light occurred in $1\frac{1}{2}$–$1\frac{3}{4}$ hours and that distal migration in dark occurred in $1\frac{3}{4}$–2 hours.

Although Herrick (1891) observed the change in position of the distal retinal pigment in living. *P. vulgaris*, Welsh (1930) was the first investigator to develop a technique for its direct measurement in living specimens. He followed the progress of both dark and light adaptation in single individuals mounted in a special chamber containing circulating sea water and found that light adaptation occurred in 40–50 minutes and dark adaptation in 80–90 minutes. However, using a technique similar to that used by Parker and involving fixation of the eyestalks before measurement, light adaptation was found to occur in 50–60 minutes and dark adaptation in $1\frac{1}{2}$–2 hours. In all these instances dark adaptation was shown to be the slower process. The fact that the rates of change from the dark-adapted to the light-adapted position and vice versa were so slow led Parker (1932) to conclude that there was little adaptive significance of these pigmentary changes except for the very general changes in illumination occurring in the day-night cycle.

It was not until the work of Kleinholz and Knowles (1938) on the shrimp, *Leander*, that it was recognized that the distal retinal pigment could be found in a position intermediate between the fully dark-adapted and light-adapted states. Resorting to the older method of measuring fixed eyestalks, these investigators found that, over a limited range of illuminations, the degree of movement of the distal retinal pigment toward the light-adapted state was directly proportional to the light intensity. In addition, it was noted that, under similar conditions of incident light, the degree of light adaptation on a black background was less than on a gray one; they concluded that this was the result of an albedo response. Their results appeared to indicate that the position of the distal retinal pigment was directly proportional to the ratio of incident to reflected light.

The following experiments were designed to extend the studies on the effect of illumination and to re-examine the influence of the background on the position of the distal retinal pigment using a technique of direct measurement on living, intact animals. This technique permits an analysis of the responses of large samples of the population with minimum disturbance of the animals being investigated.

MATERIAL AND METHODS

The animals used in the following experiments were specimens of the common prawn, *P. vulgaris*, which were collected at frequent intervals from the Eel Pond near the Marine Biological Laboratory, Woods Hole, Massachusetts. In the laboratory the animals were kept in running sea water in large aquaria. Specimens for each experiment were taken at random from this stock supply. Males and females were used indiscriminately; no differences in response between the sexes were detected. The experiments were conducted between the hours of 8:00 A.M. and 5:00 P.M. during the month of June.

In order to facilitate the collection of sufficient data to represent the typical responses of the population of prawns, a rapid method for determining the position of the distal retinal pigment was de-

veloped. This method consisted essentially of placing the prawns one by one on the stage of a dissecting microscope and measuring with an ocular micrometer and transmitted light the width of the transparent area of the distal portion of the compound eye in a direction parallel to the long axis of the stalk. It was necessary to hold the prawns in such a manner that the eyestalk was fully submerged in

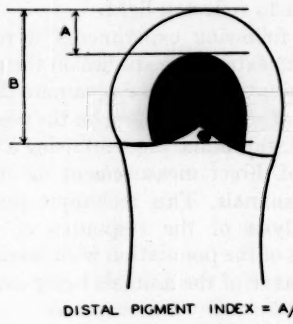


FIG. 1.—Diagram of dorsal aspect of the eye of *Palaemonetes* illustrating method of obtaining distal retinal pigment index.

sea water. This was readily accomplished with a little practice. In the fully dark-adapted eye no significant transparent area existed; the distal pigment abutted against the corneal cells. The limits of the transparent area produced by the migration of the distal retinal pigment to a more proximal position were readily apparent, providing the eye remained immersed. Since the animals varied considerably in size, it seemed advisable to express this measure of light adaptation in terms of a ratio of the width of the transparent area to the total distance from corneal surface to the proximal edge of the conspicuous pigmented black spot, or ocellus, at the base of the retina on the dorsal surface of the eyestalk (see Fig. 1).

A typical figure for the fully light-adapted state obtained in this manner would be 7/35 or a *distal retinal pigment index* of 0.20. Each ocular unit of the micrometer used in these experiments was equivalent to 26 micra. However, for the purposes of these studies absolute measurements of the position of the distal pigments were never used. This technique of direct measurement was so simple that the condition of the distal retinal pigment of three or four animals could be determined in a minute.

In order to test this method of measuring light and dark adaptation of the distal retinal pigment, five white-enameled pans, each containing ten to fifteen animals, were left in the darkroom for 3 hours. At the end of this time the distal pigment indexes of ten of the animals in one of the containers were determined and found to be zero. All the animals were maximally dark-adapted. This container and the others containing similarly dark-adapted prawns were then put into the light at an illumination of 250 foot-candles. Distal pigment indexes for ten animals were determined at approximately 15-minute intervals for the first hour using a different container of animals each time to eliminate any possible effects of repeated handling and, finally, at 95 minutes using that group of animals which was assayed for the initial determination.

At the end of a 2-hour period in the light these same five groups of animals were returned to darkness, and the course of dark adaptation was comparably followed. The distal pigment indexes were determined at 15-minute intervals until dark adaptation was complete. After the measurements had been taken on any one group, thus exposing the animals to light, this group was discarded, and a different container was used for each successive determination.

The distal pigment indexes which were obtained, first, during the process of light adaptation and, second, during the process of dark adaptation are shown in Table 1. The average distal pigment index for each time that a determination was made was calculated and is also given in this table. These values are shown graphically in Figure 2, where the average distal pigment index is plotted against time. It can be seen from this graph that the average time was about 95 minutes for light adaptation and about 60 minutes for dark adaptation.

In order to determine the influence of illumination on the position of the distal retinal pigment, a series of illuminations ranging from 4,000 to 0.0005 ft-c. was used. The two highest values, 4,000 and 2,500 ft-c., were obtained by placing containers of animals in sunlight. The remaining intensities were obtained by using incandescent lamps placed at distances ranging from 18 inches to 10 feet

TABLE 1

LIGHT AND DARK ADAPTATION OF THE DISTAL RETINAL PIGMENT OF PALAEMONETES

LIGHT ADAPTATION

15 Min.			30 Min.			50 Min.			65 Min.			95 Min.		
A	B	D.P.I.	A	B	D.P.I.	A	B	D.P.I.	A	B	D.P.I.	A	B	D.P.I.
0	40	.00	4	36	.11	6	31	.19	6	34	.18	6	30	.20
2	36	.06	3	56	.05	5	30	.17	4	25	.16	5	27	.19
4	32	.12	5	36	.14	6	29	.21	7	33	.21	5	25	.20
4	33	.12	5	36	.14	6	30	.20	7	29	.24	7	30	.23
4	37	.11	6	36	.17	6	34	.18	6	32	.19	7	27	.26
6	38	.16	3	33	.09	5	44	.11	5	32	.16	5	29	.17
0	42	.00	4	29	.14	5	34	.15	5	30	.17	7	30	.23
0	47	.00	5	33	.15	4	35	.11	5	27	.19	5	28	.18
4	35	.11	5	29	.17	7	42	.17	6	35	.17	6	35	.17
1	42	.02	4	32	.13	7	30	.23	6	26	.23	6	29	.21
	Av. .070			Av. .13			Av. .17			Av. .19			Av. .20	

DARK ADAPTATION

15 Min.			30 Min.			45 Min.			60 Min.		
A	B	D.P.I.	A	B	D.P.I.	A	B	D.P.I.	A	B	D.P.I.
4	34	.12	2	37	.05	3	34	.09	0	32	.00
2	31	.06	3	32	.09	0	34	.00	1	34	.03
4	30	.13	2	32	.06	0	33	.00	0	31	.00
5	35	.14	2	30	.07	1	32	.03	0	35	.00
3	32	.09	2	31	.06	0	34	.00	0	33	.00
2	25	.08	1	31	.03	4	35	.11	0	34	.00
3	32	.09	0	26	.00	0	30	.00	1	34	.03
3	33	.09	1	31	.03	3	35	.09	0	35	.00
5	28	.18	2	31	.06	0	33	.00	0	31	.00
3	27	.11	2	34	.06	2	34	.06	0	35	.00
	Av. .11			Av. .051			Av. .038			Av. .006	

directly above the animals. The intensities from 4,000 to 1 ft-c. were measured directly, using a Weston photometer. The intensities below 1 ft-c. were calculated, using the inverse square law. In order to obtain the lower intensities, a 15-watt lamp was painted black except for a small aperture approximately $\frac{1}{4}$ inch in diameter at the tip.

In one series of experiments white-enameled pans, with a bottom diameter of 7 inches and containing sea water to a depth of approximately $1\frac{1}{2}$ inches, were used. It was necessary to limit the number of animals in any one pan to ten to fifteen, since they tended to leap out when overcrowded. These containers were placed at the different illuminations for a period of 2 hours, after which the distal retinal pigment indexes of the prawns were determined. The total number of animals assayed for any given intensity varied from five to thirty-six, although no more than ten animals were used from any one container or in any single day.

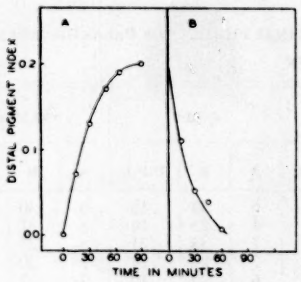


FIG. 2.—The relationship between the average distal pigment index of *Palaemonetes* and the time following transfer from darkness to light (*A*) and transfer from light to the darkness (*B*).

In a second series of experiments the responses on a black background were compared with those on a white one. Groups of animals were placed at selected light intensities in aluminum pans, similar in size and shape to the white containers, and which had previously had their internal surfaces painted with black enamel. Other groups of animals were simultaneously placed at the same light intensities in the white-enameled pans previously described. The distal retinal pigment indexes of these animals were determined after a period of 2 hours in each situation.

RESULTS

The results of the experiments on the influence of illumination on the position of the distal retinal pigment of *Palaemonetes* are summarized in Table 2. The data are arranged in such a manner that those experiments performed when only white backgrounds were used are designated as Series 1, while those experiments in which white and black were used simultaneously at selected intensities are designated as Series 2. The number of animals used at each intensity for each background is given in Table 2, and the distal pigment index given for each intensity represents an average for the number of animals indicated.

It is evident from this table that as the incident illumination of animals on a white background decreases the distal pigment index decreases. The highest indexes, indicating the maximum amount of light adaptation, occur in the range of intensities from 50 to 4,000 ft-c. of those intensities used. Between 50 and 0.0005 ft-c., the animals exhibit adaptations to the light which lie between maximum light and dark adaptation with distal pigment indexes ranging between 0.20 and 0.063. The indexes of the animals on the white backgrounds in Series 2 are consistent with those obtained in Series 1 and are appropriately inserted in Table 2 according to the light intensity used.

The average distal pigment indexes obtained for all groups of animals on white backgrounds are represented by circles in Figure 3, in which the distal pigment index is plotted against the logarithm of the illumination. The relationship is seen to be sigmoid in character.

The average distal pigment indexes for the animals on black backgrounds in Series 2 are also given in Table 2. In all cases in this series where the response on a black background was compared with the response on a white background, the average index on the black background was less than that obtained on the white background at the same intensity. These results could be expected were there an albedo response, a response to the ratio of incident to reflected light, or, in accordance with the results described above, were the black background acting simply by reducing the amount of light striking the eyes.

TABLE 2

RELATIONSHIP BETWEEN RETINAL PIGMENT INDEX AND ILLUMINATION USING WHITE AND BLACK BACKGROUNDS

Series	Light Intensity (Ft-c.)	No. of Animals	Average Distal Pigment Index	
			White Background	Black Background
1	4,000	5	.21	.....
1	2,500	9	.20	.....
1	250	18	.19	.....
2	200	10	.22	.....
2	200	10	.....	.16
1	100	8	.17	.....
1	50	8	.20	.....
2	50	10	.22	.....
2	50	10	.....	.16
2	45	10	.17	.....
2	45	10	.....	.13
1	25	36	.18	.....
2	25	10	.20	.....
2	25	10	.....	.15
1	10	8	.19	.....
2	10	10	.19	.....
2	10	10	.....	.12
1	5	8	.17	.....
1	2	26	.16	.....
2	2	10	.18	.....
2	2	10	.....	.10
1	1	28	.17	.....
2	1	10	.18	.....
2	1	10	.....	.073
2	.5	17	.16	.....
2	.5	20	.....	.076
1	.12	15	.12	.....
2	.05	20	.13	.....
2	.05	18	.....	.061
1	.01	20	.067	.....
1	.0026	20	.077	.....
1	.001	20	.070	.....
1	.0005	20	.063	.....

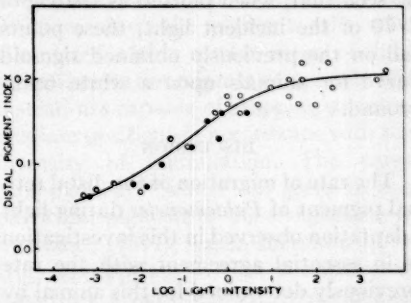


FIG. 3.—The relationship between the average distal pigment index of *Palaemonetes* and the logarithm of the light intensity after a 2-hour period of adaptation (see text for further description).

In order to distinguish between these two possibilities, the difference between the amounts of light reflected from the black and the white backgrounds was determined, using a Weston photometer. It was found that the black background diffusely reflected about 1/70 of the amount of light reflected from the white one. The average distal pigment indexes obtained on the black background were therefore plotted in Figure 3, not according to the log of the illumination for animals upon a white background, but instead as the log of 1/70 of this value. It was obviously not necessary to plot either the black or the white background values in terms of the actual amount of reflected light; the white background diffusely reflected approximately 60 per

cent of the incident light, and on a logarithmic plot this would constitute an insignificant shift of all points to the left. In addition, the relationship between the values obtained from the animals on the two backgrounds remains the same whether incident or reflected light is used.

The average indexes obtained for the animals on the black background are plotted in Figure 3 as solid circles. It can be seen that, when plotted as the log of 1/70 of the incident light, these points fall on the previously obtained sigmoid curve for animals upon a white background.

DISCUSSION

The rate of migration of the distal retinal pigment of *Palaemonetes* during light adaptation observed in this investigation is in essential agreement with the rate previously determined for this animal by Parker (1897) and slower than that observed by Welsh (1930). Dark adaptation was found to occur more rapidly than that reported by either Parker or Welsh. Both of these investigators found dark adaptation to be the slower process. In contrast it was found in the studies reported here that light adaptation was the slower process.

It is of interest in this regard to know whether the distance covered in the distal pigment migration in both investigations is comparable. In Welsh's experiments on light adaptation the maximum distance migrated by the distal retinal pigment of one animal was 175.4 micra. In the dark adaptation the maximum distance migrated was 217.1 micra. In the experiments reported here the average pigment migration distance of fifty animals was 152.8 micra. The difference between the absolute distance of migration in Welsh's experiments and these can probably be accounted for by differences in sizes of the animals used. However, in comparing the distance migrated in the light adaptation experiments, it is evident that in Welsh's experiment it took less time (40 minutes) to migrate a greater distance (175.4 micra) than it did in the experiments reported here (152.8 micra in 95 minutes).

The experiments reported on here were done at a temperature of 23 $\pm$ 1° C., and, although Welsh reports 20° C. for only the dark adaptation experiment, it is probable that the light adaptation was also done at approximately 20° C. It is unlikely, however, that temperature differences can account for the differences in rates found between the present work and that of Welsh, especially in view of the reversal of comparative rates of dark and light adaptation.

It is probably of greater importance to recognize that the results indicated here represent averages of fifty animals, many more than in methods which have been used previously. It is apparent from examination of Table 1 that there is substantial variation among the animals in the length of time taken for the pigment migration. Some animals are capable of achieving the maximum light-adapted state as indicated by a distal pigment index of 0.20 to 0.22 in approximately 50 minutes, while others have not quite arrived at this condition in 95 minutes. It is believed that the use of the distal pigment index, which takes into consideration variations in the size of the eye, and averaging the response of a large sample of prawns, yields a more duplicatable picture of the distal retinal pigment activities than methods which have been used previously.

In the experiments reported here illuminations ranging from 4,000 to 0.0005 ft-c. were used. Throughout this range the distal pigment index varied with the light intensity. The steepest portion of

the curve exhibiting the relationship was between the intensities of 0.01 and 10 ft-c.—a thousand-fold difference in illumination. This indicates that the distal pigment of *Palaemonetes* is highly responsive to illumination and that the amount of light which enters the eye is precisely regulated over a relatively large range of illuminations. Seven log units are shown in these experiments. This supports the view that the distal retinal pigment is of greater functional significance in regulating the amount of light admitted to the retina than formerly believed.

In the experiments reported here the more proximal position of the distal pigment on a white background than on a black background, other factors being equal, confirmed the observations of Kleinholz and Knowles (1938). However, it was found that this response was not an albedo response, as reported by the latter workers, but instead merely a response to the lowered illumination caused by the black background. This conclusion is not surprising when one considers the predominant source of light reaching the eyes of the prawns under such conditions. The pans used in the experiments were 3 inches deep and contained approximately 1½ inches of sea water. The light sources used were relatively small, while the inner surfaces of the containers were relatively large, diffuse reflectors. A prawn in such an environment has its eyestalks projected laterally with the retina free from any significant shielding effect of the body. The light source proper stimulates an extremely small number of ommatidia in comparison to the number reached by the reflected rays of light both directly from the background and indirectly by internal reflections from the water-air interface. Therefore, the light reaching the eye directly from the source has, as expected, very minor influence in determining the general position of the distal retinal pigment.

The distal retinal pigment cells of *Palaemonetes* are capable of an adaptive response in that the amount of light entering the eye can be rather precisely regulated by the position which the pigment assumes in response to a wide range of illuminations. It is not an all-or-nothing reaction, since the pigment cells once stimulated within a wide range of light intensities do not migrate completely but instead are capable of stopping at intermediate positions in accordance with the intensity of illumination. The name "iris pigment," which was given to this particular pigment of the compound eye by Exner (1891), seems therefore to be truly appropriate in that it appears to constitute a light-regulating mechanism comparable to the vertebrate iris.

SUMMARY

1. The distal retinal pigment of *Palaemonetes vulgaris* is very sensitive to illumination. Between 0.0005 and 50 ft-c. the position of this pigment is a function of the intensity of illumination. A maximum light-adapted state is observed between 50 and 4,000 ft-c.

2. At any given intensity of incident illumination the pigment is in a more dark-adapted position on a black than on a white background. This is not a true background or albedo response but simply a response to the brightness of the visual field.

3. These results suggest that mechanical movements of the distal retinal pigment are of greater functional significance than formerly believed in the regulation of the amount of light reaching the retinula cells of the eye.

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ALTERATIONS IN THE CAPACITY FOR LIGHT AND DARK ADAPTATION OF THE DISTAL RETINAL PIGMENT OF PALAEMONETES[1]

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EARLY investigations on the regulation of retinal pigments of crustaceans concerned the degree of independence between the two eyes of an individual. Parker (1897), Castle (1927), and Bennitt (1932), working on *Palaemonetes*, and Knowles (1950), working on *Leander*, covered one eye and exposed the other to light. Bennitt also examined several species of crustaceans with opaque exoskeletons in which transmission of light through the exoskeleton might be avoided. The results were not in every case conclusive, but Bennitt and Knowles agreed that the pigment movements in the two eyes were interrelated and suggested the possibility of a hormonal mechanism of control.

Kleinholz (1936) produced direct evidence of hormonal control when he found that injection of eyestalk extract into dark-adapted *Palaemonetes* resulted in migration of the distal and reflecting pigment to the light-adapted position. Injection of the extract into light-adapted animals effected no change in position of the pigment. The fact that eyestalks of light-adapted shrimp were more effective than those of dark-adapted animals in bringing about light adaptation suggested that the substance extracted from the eyestalks was normally involved in control of the pigment migration. Kleinholz also demonstrated the presence in the eyestalks of several other decapod crustaceans of a substance effective in producing light adaptation in *Palaemonetes*.

Welsh (1939) showed that extracts of crayfish eyestalks, injected into dark-adapted *Cambarus*, brought about light adaptation of the distal pigment and that higher concentrations caused light adaptation of the proximal pigment also. In 1938 Kleinholz and Knowles reported that *Leander* showed graded responses to injections of graded concentrations of eyestalk extract. Welsh produced evi-

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dence that the sinus gland was an important source of the substance effective in causing retinal pigment migration in *Cambarus*.

In 1948 Smith reported that the removal of the sinus glands from several species of grapsoid crabs failed to result in complete dark adaptation. In the apparent absence of the sinus gland some of his operated animals were capable of normal responses to light and darkness. Some also showed a persistent rhythm in constant darkness. In the same paper Smith also reported that extracts of the optic ganglia, supraesophageal ganglia, and thoracic cord, as well as the sinus gland, were effective in bringing about light adaptation in the crabs studied.

Kleinholz (1949) reported that the proximal pigment of crayfish responded to light and darkness in the absence of the sinus gland. A similar situation was found for *Leander* by Knowles (1950).

MATERIAL AND METHODS

The animals used in these experiments were *Palaemonetes vulgaris*, which were readily collected in large numbers in the Eel Pond at Woods Hole, Massachusetts. They were maintained in the laboratory in running sea water in large aquaria. None of the prawns used in the experiments had been in the laboratory for more than 4 or 5 days.

The state of the distal retinal pigment was readily determined in a quantitative manner by viewing the eyes in the living, intact animals with the aid of a binocular dissecting microscope possessing an ocular micrometer. The magnification used in most of the work was 40×. The technique has been described in an earlier report (Sandeen and Brown, 1952).

The method that was used for determining the state of the distal pigment at any given moment under specific conditions involved getting the average distal pigment index for ten animals. These data could be obtained in about 3 minutes. When it was desired to follow the changes which occurred over a period of time when the prawns were subjected to certain experimental conditions, as many groups of ten animals as the particular experiment required were placed in white-enameled pans and subjected to the particular conditions. At each selected interval one of the pans of animals was assayed for the average state of the distal pigment and then discarded. In this manner any possible alteration in the state of the pigment due to handling was minimized. That this sampling technique was fully justified will become quite evident in observing the smooth trends in the changes which occur under the conditions of the various experiments which were performed.

EXPERIMENTS AND RESULTS

RELATIONSHIP BETWEEN TIME IN DARKNESS AND RATE OF READAPTATION TO LIGHT

In the course of experimentation with the processes of light and dark adaptation of the distal retinal pigment of *Palaemonetes*, it became increasingly evident that the ability to dark-adapt was considerably influenced by the light history of the animals and, under some conditions, by a persistent diurnal rhythm. In the following experiments attempts were made to discover to what extent, and in what manner, the ability to light- and dark-adapt was influenced by the length of time the animals had previously been in darkness or light, respectively.

To maintain the diurnally rhythmic factor as constant as possible, the prawns used in these experiments were taken directly from the stock aquaria in the laboratory, where they were subjected to daily fluctuations in illumination ranging

from extremely low illumination at night to about 10–20 foot-candles during the brightest hours of the day. All the experiments were performed between the hours of 6:00 A.M. and 5:00 P.M. It had been established that the animals taken from these tanks at these hours were able to dark-adapt at what appeared to be a maximal rate. During the experiments

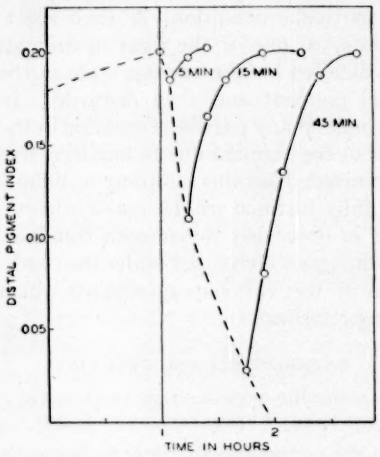

FIG. 1.—The relationship between time in darkness and rate of light adaptation upon return to an illumination of 250 ft-c.

the animals were kept in circular white-enameled pans, having a bottom diameter of 7–8 inches, in 1–1½ inches of water.

In the first series of experiments fifteen white-enameled pans containing ten animals each were placed in an illumination of 250 ft-c. and the animals permitted to adapt to this situation for 1 hour. At the end of this time the average state of the distal retinal pigment was determined for one pan, and then all fifteen pans were placed in darkness. At the end of 5 minutes five of the pans were returned to 250 ft-c.; after 15 minutes five more of the pans, and after 45 minutes the last five, were returned to the 250 ft-c. illumination. The average state of the distal retinal pigment of these animals was determined at the time of return to the light, and at 10, 20, 40, and between 60 and 75 minutes thereafter by determining the average state of the distal pigment in a different pan at each of these times.

The results of this experimental series are plotted in Figure 1. It is readily seen that the rate of dark adaptation is rapid and that there is no significant difference in the rates of light adaptation following return to light among the 5-, 15-, and 45-minute samples.

RELATIONSHIP BETWEEN TIME IN LIGHT AND RATE OF READAPTATION TO DARK

In a second series of experiments fifteen white-enameled pans containing ten animals each were placed in darkness and permitted to remain there for the animals to dark-adapt during the course of 1 hour. At the end of this time all fifteen pans were restored to light at 250 ft-c., and ten animals from one pan were examined immediately to determine the average state of the distal pigment at this time. At the end of 5 minutes five of the pans were returned to the darkroom, five more were returned after 15 minutes, and the last five after 45 minutes. At the time of return of each group to darkness a determination of the average distal pigment state was made by examining the animals of one pan. The state of the pigment at 10, 20, 45, and 60 minutes following return to darkness was made by using at each time one of the pans of ten prawns as a sample of the population and then discarding it.

It is evident from the results obtained in this series of experiments (Fig. 2) that dark adaptation had become complete within 1 hour and that subsequent light adaptation was occurring at essentially

the same maximal rate that was observed following the three stages of dark adaptation in the first experimental series. But now, when one compares the behavior of the distal pigment upon return to darkness, striking differences are observed which are a function of the time the prawns had previously spent in light. After only 5 minutes in light the animals continued during the succeeding 10 minutes in darkness to light-adapt at a rate virtually that of the animals remaining during that time in light. And after that the rate of dark adaptation was less than maximal. Those animals left for 15 minutes in light exhibited less tendency, but nonetheless a real one, to continue light-adapting in darkness and afterward a definitely submaximal rate of dark adaptation. After 45 minutes in light the animals, upon return to darkness, no longer showed the tendency to continue light-adapting upon return to darkness and, furthermore, now exhibited a definite return toward the original rapid rate of dark adaptation, though it is still at this time submaximal.

It is apparent from the preceding experiments that prawns which have been in darkness for a time and are then exposed to light are stimulated to initiate a light-adapting process which will proceed for an appreciable time even though the light stimulus is removed. This is the character of response one might expect to obtain if the light stimulus resulted in the liberation of a blood-borne light-adapting hormonal factor. Were such a factor responsible for the phenomenon described here, it would be necessary to conclude that the duration of action of the light-adapting principle was such as to bring about extensive light adaptation. But even in continued illumination the concentration in the blood becomes rapidly reduced.

Returning now to the results of the first series of experiments, it is evident that the light-adapting principle can be rapidly liberated in response to continued light at any time following a shorter or longer sojourn in darkness.

These results, furthermore, strongly support the hypothesis of a dark-adapting hormone normally responsible for dark adaptation. The animals in the second series of experiments are behaving quite as one would expect them to do had there been a temporary exhaustion of the sources of dark-adapting hormone during the initial hour-long period of dark adaptation. Following the different intervals in light, after the action of the light-adapting principle has passed its maximum of activity, the animals, being in darkness, might be expected to return to the dark-adapted state in the usual minimum of time were only a light-adapting hormone operative. This they appear unable to do. However, the longer the time in light, the more rapidly are they able

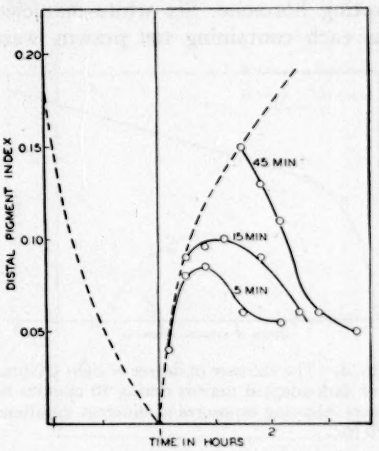

FIG. 2.—The relationship between time in light at 250 ft-c. and rate of re-dark-adaptation following return to darkness.

to re-dark-adapt. They are behaving as if they were, during this time, recharging their sources of dark-adapting principle.

RELATIONSHIP BETWEEN DURATION OF A 250-FT-C. FLASH AND AMOUNT OF SUBSEQUENT LIGHT ADAPTATION

In the next experiment an attempt was made to determine the minimum duration of light stimulus at 250 ft-c. for this initial extensive discharge of light-adapting hormone. Six white-enameled pans each containing ten prawns were placed in darkness. Two hours later, after dark adaptation was complete, the first pan was given a 5-second light flash of 250 ft-c. and then returned to darkness. The next pan was given a 15-second, the third a 30-second, the fourth a 60-second, the fifth a 120-second, and the last a 300-second flash, before being returned to darkness. The average state of the distal pigment was then determined for each pan of animals 10 minutes after the beginning of the light stimulus. These results are plotted in Figure 3. It is readily apparent that practically no light adaptation has occurred following the 5-second stimulus but that the light-adapting response increases very steeply with longer periods up to about 1 minute, when the rate of increase has dropped to a low and constant level. In brief, it appears that, at this intensity of illumination, 1 minute of light is an adequate stimulus for maximum discharge of any stored light-adapting hormone but that the light continuing beyond this time is responsible for a slower and steady liberation of the hormone.

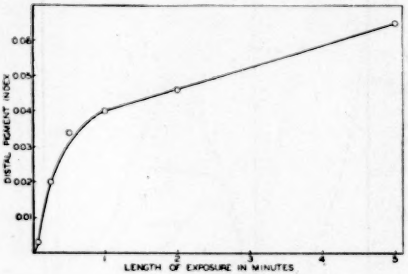

FIG. 3.—The increase in degree of light adaptation of dark-adapted prawns during 10 minutes in darkness following exposures of different durations at 250 ft-c.

INFLUENCE OF PREVIOUS LIGHT HISTORY ON RESPONSE TO A 1-MINUTE LIGHT FLASH

If *Palaemonetes* are removed from the stock aquaria during the daytime, placed in darkness, and permitted to become and remain dark-adapted over a period of 3 or 4 hours, they will respond to a 1-minute, 250-ft-c. flash of light by a transitory movement of the distal retinal pigment toward the light-adapted state. The response far outlasts the duration of the stimulus. Under these described conditions the distal pigment typically reaches some intermediate degree of light adaptation during the succeeding half-hour in darkness and then more or less rapidly returns to a state of complete dark adaptation. It is, therefore, evident that a 1-minute light flash will produce a light-adaptational response which requires more than an hour and a half to subside.

If, on the other hand, the prawns are allowed to remain overnight in darkness and then are submitted to a 1-minute, 250-ft-c. flash at about 6:00 A.M., the response to the flash is quantitatively quite different from that just described. Under these conditions the degree of light adaptation subsequently attained in darkness following the flash is significantly greater and often does not reach a maximum in less than an hour. Moreover, the return to dark adaptation is extremely slow, occurring gradually over a period of 6–9 hours. This is illustrated in Figure 4, *C*,

where, for comparison, the rates of dark adaptation at the same time of day of animals taken from two laboratory illuminations are included (*A* and *B*). Increasing the duration of the exposure to the 250-ft-c. illumination from 1 minute to 30–45 minutes did not increase the ability of the prawns to return to the dark-adapted condition following the return to darkness.

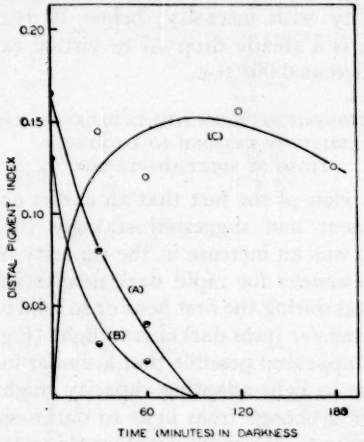

FIG. 4.—*A* and *B*, dark adaptation of prawns transferred from a higher and lower illumination of the laboratory to darkness at 6:00 A.M. *C*, response of animals kept in darkness overnight to a 1-minute, 250 ft-c. flash at 6:00 A.M.

INFLUENCE OF REPEATED 1-MINUTE LIGHT FLASHES IN LIGHT ADAPTATION

Further evidence that the 1-minute light flash at 250 ft-c. had temporarily depleted an accumulation of a light-adapting hormone was seen from another type of experiment in which some dark-adapted prawns were subjected to a single 1-minute flash, others to two 1-minute flashes 1 minute apart, and still others to three 1-minute flashes with intervening 1-minute periods of darkness. In all three of these groups there was a continuing light adaptation, reaching a maximum at about 30 minutes in darkness; and there was no significant difference among them in the degree of light adaptation achieved or in its duration. The second and third stimuli, though striking the eye before any appreciable light adaptation of the distal pigment was evident, were without significant added effect upon the light-adapting process.

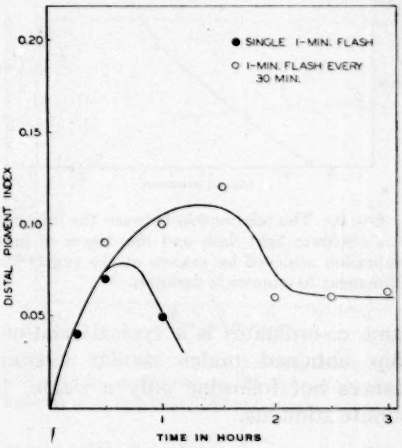

FIG. 5.—Comparison of the effects of subjecting prawns in darkness to a 1-minute, 250 ft-c. flash every 30 minutes, with the effect of a single initial flash.

In another experiment seven groups of ten animals were placed in darkness, and, after dark adaptation had become complete, these were given a 1-minute, 250-ft-c., flash of light every 30 minutes. The results of this treatment are graphically depicted in Figure 5. Here it is evident that there was an additive effect of the stimuli for the first 90 minutes or so but thereafter a drop to an equilibrium state of intermediate adaptation of the pigment. This form of relationship is what one would expect were a substantial amount of the hormone produced in re-

sponse to the initial stimulus such as would reach a maximum of response in 30–60 minutes, and then each succeeding stimulus resulted in a constant but significantly smaller discharge of light-adapting hormone. Plotted upon the same co-ordinates is a typical relationship obtained under similar circumstances but following only a single, 1-minute stimulus.

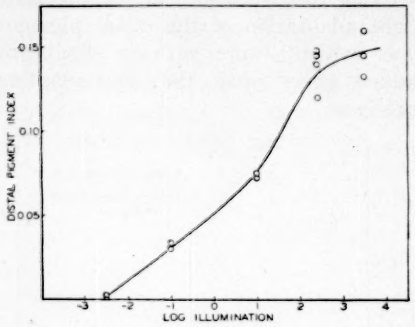

FIG. 6.—The relationship between the intensity of a 1-minute light flash and the degree of light adaptation achieved by prawns at the end of the subsequent 30 minutes in darkness.

RELATIONSHIP BETWEEN INTENSITY OF A 1-MINUTE LIGHT FLASH AND AMOUNT OF SUBSEQUENT LIGHT ADAPTATION

Another experimental series was devised to discover the relationship between the intensity of the light stimulus and the magnitude of the response of the distal pigment. For this the duration of the stimulus was kept constant at 1 minute. A series of illuminations ranging from 0.003 to 3,000 ft-c. was used. The animals were taken from the stock aquaria, some in the morning and some at noon, and placed in darkness. After 3–4 hours the animals, in groups of ten in white-enameled pans, were given the 1-minute light stimuli and returned to darkness. Each group of animals was examined for the average state of the distal pigment after 30 minutes in darkness. The results are illustrated in Figure 6. Each point on the curve is the average of one group of ten animals. It can be seen from this relationship that for intensities above about 100 ft-c. there is not a great deal of change with intensity; between 10 and 100 ft-c. there is a rapid change in activity with intensity; below 10 ft-c. there is a steady drop off to virtual extinction at 0.003 ft-c.

RELATIONSHIP BETWEEN TIME IN DARKNESS AND ABILITY TO RESPOND TO A 1-MINUTE FLASH BY LIGHT ADAPTATION

In view of the fact that an earlier experiment had suggested strongly that there was an increase in the capacity of *Palaemonetes* for rapid dark adaptation at least during the first hour or so following transfer from darkness to light (Fig. 2), it appeared possible that a similar increase in light-adapting capacity might follow a change from light to darkness. In order to determine whether this were the case, prawns were taken from the stock aquaria of the laboratory and placed in white-enameled pans ten animals in each. All were placed in the darkroom. After different intervals of time, groups of these were subjected to a 1-minute, 250-ft-c. flash and then returned to darkness.

Five groups of ten animals were left in darkness for 5 minutes. At the end of this time all were subjected to the light flash. One group was assayed immediately for the average state of the distal retinal pigment; the other four groups were returned to darkness. These were assayed at 30 minutes and 1, 2, and 3 hours thereafter.

A second lot of five groups of ten animals was left for 15 minutes in darkness, a third for 30 minutes, a fourth for 45 minutes, a fifth for 1 hour, a sixth for 2 hours, a seventh for 3 hours, and an

eighth for 14 hours (from the preceding evening). As with the first group, these were subjected to the 1-minute, 250-ft-c. flash at the end of their exposure to darkness, and one of the five groups of ten assayed immediately. The remainder in each case were returned to darkness and the distal pigment state determined at 30

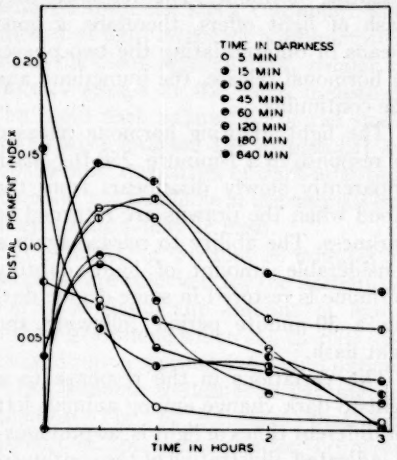

FIG. 7.—Responses of the distal pigment to a 1-minute, 250 ft-c. flash administered to prawns following various intervals in darkness.

minutes, and 1, 2, and 3 hours afterward by using one of the pans of animals as a sample at each of these times.

The results obtained with all these eight series are illustrated in Figure 7, in which the distal pigment indexes at the time of the flash, and at the four times thereafter, are shown. If one now recalls the normal rate of dark adaptation of prawns taken from the stock tanks at this time of day (see Fig. 1), it will be readily evident that the rate of dark adaptation of the animals given the light flash only 5 minutes after being placed in darkness is almost the normal one; it is only slightly retarded. It is evident, however, that the magnitude of effect of the 1-minute flash becomes progressively greater as the animals are left for longer and longer times in darkness prior to it. The effect of the flash is greatest in those animals which had been in darkness for 14 hours. While this is obviously in largest measure due to variation in the initial discharge of light-adapting principle, reciprocal alteration in amount of a dark-adapting factor may also be concerned.

The following procedure was used to see better the gradual alteration in re-

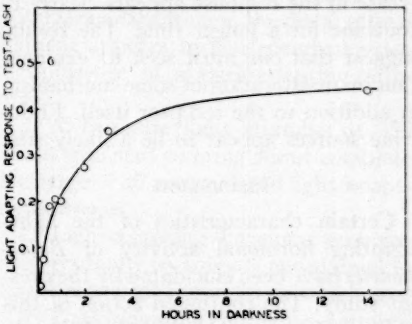

FIG. 8.—The relationship between time in darkness and the magnitude of the light-adaptational response to a 1-minute, 250 ft-c. flash.

sponse with time in darkness: the difference between the experimental values and the values obtained from animals placed in darkness at the beginning of the experimental period, but receiving no 1-minute flash, were obtained at 30 minutes and 1, 2, and 3 hours for each of the experimental series. The sum of these was considered to be a fair measure of the effect of the 1-minute flash. In Figure 8 these sums are plotted against the times of previous exposure to darkness. Here it is quite evident that there is, following the transfer of animals to darkness, first a very rapid increase in the degree the animals light-adapt. This rate of increase of the total response gradually declines, and after 4–6 hours the increase continues at a very low rate. The initial rapid increase in capacity to respond to

the light flash may in some measure be explained in terms of mechanical adaptation of the eyes through retinal pigment movement and to adaptation of the basic photoreceptive mechanism. Such changes would be expected to result in a rapid increase in the sensitivity of the eye to the test flash. It seems highly improbable, however, that these changes would require more than 1 or 2 hours at most for their completion; yet the gradual increase in the response appears clearly to continue for a longer time. The results suggest that one must seek its explanation in an alteration in some mechanism in addition to the receptor itself. Endocrine sources appear to be a likely site.

DISCUSSION

Certain characteristics of the light-adapting hormonal activity of *Palaemonetes* have been elucidated by the present study. The continued action of this hormone for a variable length of time in darkness following cessation of the stimulus for its release offers a valuable means of measuring variation in ability to light-adapt. The pretreatment of prawns appears to have little effect upon the rate of light adaptation when the animals are permitted to remain in light during the process, as the prawns light-adapt at a rate characteristic of the stage of dark adaptation which has occurred when the dark-to-light change is made. The curves for light adaptation may be superimposed (Fig. 1), showing that the response of the animals to this stimulus depends upon the state of the retinal pigments at the time of change.

The continuation of the process of light adaptation which occurs when the prawns are returned to darkness following brief periods in light is a clear indication of the rapid release of a considerable quantity of the light-adapting hormone in response to the abrupt dark-to-light change. At 250 ft-c., a 1-minute exposure apparently results in the maximum immediate release of the light-adapting hormone. Complete light adaptation, such as that which occurs when the animals are maintained in an illumination of moderate intensity, must result from a continued slower production of the light-adapting hormone. The response to a flash of light offers, therefore, a good means of differentiating the two phases of hormonal release, the immediate and the continuing.

The light-adapting hormone released in response to a 1-minute, 250-ft-c. flash apparently slowly disappears from the blood when the prawns are returned to darkness. The ability to release again a considerable amount of light-adapting hormone is restored in some degree during a 30-minute period following the light flash.

The variations in the response to a light-to-dark change among animals left for different times in light is, as previously indicated, illustrative of the continued action for a certain time of the originally released light-adapting hormone. The rates of readaptation to dark vary significantly among the groups. It is assumed that, if no opposing forces were operative, the rate of disappearance upon return to the dark would, at any given moment, be simply a function of the concentration of light-adapting hormone. This is not the situation. It seems more reasonable, therefore, that the variations observed can best be explained in terms of a second hormonal substance which is effective in bringing about dark adaptation. The ability to secrete dark-adapting hormone must increase at least for a time as the animals are maintained for longer periods in the light and must be the predominant factor in determining the rate of dark adaptation.

It is obvious (Fig. 4) that comparable

states of light adaptation, such as exist between stock animals just put into the dark and the dark-maintained animals 60 minutes following a flash, may be followed by wide variation in ability to readapt to dark. The dark-maintained animals appear to have no ability for immediate dark adaptation, unlike the animals removed from the stock container. The amount of light-adapting hormone present in the blood is presumably similar in the two groups at the times indicated. The rapid dark adaptation of the stock group must indicate the availability of considerable quantities of dark-adapting hormone, not available in the animals exposed to the 1-minute flash.

It is seen (Fig. 5), that 30 minutes of darkness are sufficient for the accumulation of a significant amount of light-adapting hormone which can then be discharged upon appropriate stimulation.

The response to flashes of varying intensities (Fig. 6) indicates that the amount of the light-adapting hormone released is determined by the intensity of the stimulus. This would support the view that the graded response of the distal retinal pigment to graded intensities of illumination (Sandeen and Brown, 1952) depends in part upon differing amounts of a light-adapting hormone in the blood.

SUMMARY

1. Evidence is presented supporting the view that the light adaptation of the distal retinal pigment of *Palaemonetes* following a dark-to-light change is due to an initial large discharge of an accumulated light-adapting hormone followed by a continuing lower rate of secretion.

2. Dark adaptation of the distal retinal pigment appears to be dependent upon the presence of a dark-adapting principle.

3. A 1-minute light stimulus at 250 ft-c. is sufficient to bring about complete discharge of all accumulated light-adapting hormone.

4. Light-adapting hormone accumulates gradually in animals maintained in darkness to a maximum in about 6 hours.

5. Dark-adapting hormone accumulates gradually, at least for a brief time, in darkness.

6. Evidence is submitted in support of the hypothesis that graded light adaptation of the distal pigment in response to graded illumination is normally determined, at least in part, upon quantitative production of a light-adapting hormone.

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SENSITIVITY OF FISH TO LIGHT AND THE LATERAL LINE SYSTEM[1]

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IN THE Moser River, Nova Scotia, Huntsman (1942) observed that salmon, *Salmo salar*, failed to respond to light at very high temperatures; parr which normally avoid the light migrated into shallow water, and the largest salmon came to the surface instead of going farthest down as is their normal behavior.

Andrews (1946) observed, under field and experimental conditions, that the response of certain fish to light varies both with age and with change in light intensity. Suckers, *Catostomus commersonii*, which are more than one year old, for example, avoid light but will leave cover when the intensity is low under such conditions as starlight, moonlight, and late twilight. Underyearlings, however, seek the light in all light intensities, ranging from starlight to bright sunlight. This is in conformity with the vertical distribution of this species in nature, underyearlings being found near the surface or in shallow water and the larger fish in successively deeper waters where the light intensity is less, since light is increasingly absorbed with increasing depth.

At very high temperatures, however (approaching the lethal), it was found (Andrews, 1946) that the fish no longer responded to light; instead, as Huntsman noted, they became insensitive to it and distributed themselves at random, often with much increased activity. The temperature at which fish became insensitive to light was found to vary with the age of the fish, the light intensity, and the temperature of acclimation.

This phenomenon of insensitivity, or failure to respond to light at high temperatures, has been investigated further in an attempt to explain its nature from a physiological viewpoint.

Rubin (1935) studied thermal reception in the sunfish, catfish, and mummichog. His observations indicated that certain receptors supplied by the lateral line nerve are concerned with thermal reception. Assuming that heat is the dominant stimulus at the high temperature and that it results in the increased activity and failure to respond to light, then, if the heat impulses can be prevented from reaching the central nervous system, the fish should continue to respond to light at higher temperatures than those found for normal fish. In other words, cutting the path of the heat impulse to the central nervous system should result in a continuation of the response to light and at temperatures nearer the lethal than is the case where the path is not cut.

MATERIAL AND METHODS

Goldfish, *Carassius auratus*, were used as experimental material. These were obtained from a local dealer in New York City and stored in laboratory aquaria.

[1] These experiments were conducted in the Washington Square College of Arts and Science, New York University, and the writer wishes to express sincere thanks and appreciation to Dr. H. A. Charipper, chairman of the Department of Biology, for the facilities which made possible the results obtained.

One group was kept at room temperature or about 24° C. and another at 12° C.

The method and technique employed in the following experiments were but a slight modification of that used in a previous paper (Andrews, 1946). The fish were placed in a round black glass chamber measuring 9 inches in diameter and 3 inches in depth; this in turn was placed in an aquarium as shown in Figure 1. The chamber was held in place and supported by a wire stand. A black cardboard cover was attached to the top of the aquarium, so that one half of the chamber was covered while the other half was exposed to direct light. The aquarium was filled with water and fitted with a system of heaters, so that the temperature of the water in the chamber in which the fish was placed increased at about 1° C. every 5 minutes. A 15-watt electric light was suspended at 2 feet above the chamber. The light intensity at the surface of the water in the chamber was 5 candle power.

A single goldfish was placed in the chamber, and after about 15 minutes one half was exposed to direct light. The period of exposure was 15 seconds, during which time observations were made, and this was followed by 15 seconds of darkness during which time the cover was switched to the alternate half of the chamber, followed by a second exposure to light, etc. About ten observations were made at each degree of temperature increase. The position of the goldfish in the chamber was determined by use of a mirror suspended above the chamber.

In bright artificial light the lateral line nerve in the goldfish can be seen lying just beneath the skin and above the lateral line canal. A few scales were removed about 5 mm. posterior to the gills, and a vertical razor-blade incision was made through the skin until contact was made with the nerve, which, in turn, was cut. The operation was then repeated on the opposite side. In the control fish the incision was made as above, but the nerve was not cut.

EXPERIMENTAL RESULTS

In the following experiments goldfish took cover and continued to do so, with increase in temperature, until a point was

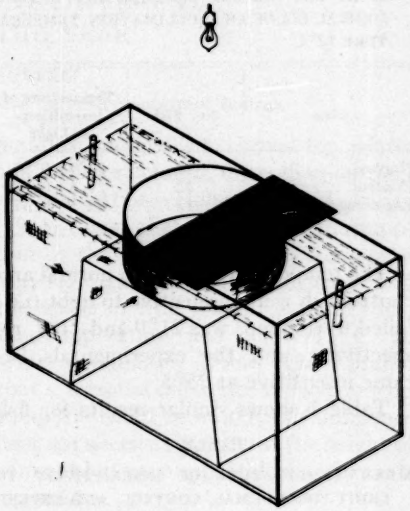

FIG. 1.—Experimental apparatus (see text for explanation).

reached when they failed to respond to the light stimulus. They were then said to be insensitive to light.

Two groups of fish were used. Previous to observation one group was kept in running water of 12° C., and the other was kept in aquaria at room temperature of about 24° C. It is presumed that the two groups were acclimated to 12° and 24°, respectively, since they were living under these temperature conditions for at least two weeks before observations were made.

For experimental purposes each group

was divided into normal, control, and experimental fish. In the experimentals the skin and lateral line nerve were cut; in the controls only the skin was cut; and in the normal fish no cut was made at all.

The results for goldfish acclimated to 12° are shown in Table 1.

TABLE 1

MEAN TEMPERATURE OF INSENSITIVITY TO LIGHT FOR NORMAL CONTROL AND EXPERIMENTAL GOLDFISH. ACCLIMATION TEMPERATURE 12° C.

Class	No. Fish	Temperature of Insensitivity to Light
Normal	12	21°.9 ± 0°.30
Control	12	21.4 ± 0.60
Experimental	12	25.5 ± 0.37

The temperature at which normal and control fish were insensitive to light (i.e., failed to respond) was 21°.9 and 21°.4, respectively, and the experimentals became insensitive at 25°.5.

Table 2 shows similar results for fish acclimated to room temperature of about 24°.

TABLE 2

MEAN TEMPERATURE OF INSENSITIVITY TO LIGHT FOR NORMAL, CONTROL, AND EXPERIMENTAL GOLDFISH. ACCLIMATION TEMPERATURE ABOUT 24° C.

Class	No. Fish	Temperature of Insensitivity to Light
Normal	8	30°.4 ± 0°.59
Control	5	29.4 ± 0.43
Experimental	8	33.9 ± 1.17

Here again a significant difference between normal and control as distinct from experimental fish can be noted. Whereas normal and control fish became insensitive at 30°.4 and 29°.4, respectively, the experimentals did not become insensitive until a mean temperature of 33°.9 was reached.

DISCUSSION

The results indicate that the response to light of goldfish is upset by high temperature. It thus appears that a point is reached when the heat stimulus is so strong that it determines the response of the fish. This is manifested by its increased activity and failure to respond to a presumably weaker stimulus, such as the light intensity used.

Cutting the path by which the heat impulses reach the central nervous system shows that the normal response to light can be restored, since fish continue to respond to light at temperatures well above that found for normal and control fish.

It is conceivable, too, that sensitivity to light may be restored at a temperature still closer to the lethal. For example, heat impulses may have sensory paths connected with the lateral line nerve anterior to the cut, and other paths may not be connected with the lateral line nerve at all. Cutting these paths may have an increased effect.

SUMMARY

1. Experiments have been performed which show that fish no longer sensitive to light at high temperatures may have that sensitivity restored by cutting the lateral line nerve.

2. The restoration and increase in sensitivity to light are attributed to the fact that the heat stimuli received by the temperature receptors distributed along the lateral line system are prevented from reaching the central nervous system. Then, since heat is no longer the dominating stimulus, the fish continues to respond to light until a point nearer the lethal is reached.

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THE OXYGEN CONSUMPTION OF TWO SPECIES OF SALAMANDERS AT DIFFERENT SEASONS OF THE YEAR[1]

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IT IS well known that the metabolic rate of poikilothermous animals changes directly with temperature and that a decreased rate of oxygen consumption reflects a lessened state of activity by the organism. However, the influence of temperature on metabolism varies greatly among the various species of cold-blooded vertebrate animals. Gardner and King (1922), in comparing the metabolism of the goldfish, *Carrasius auratus*, and the trout, *Salvelinus fontinalis*, found that the trout are much less sensitive to temperature changes. In the field of reptilian physiology, Benedict (1932) reported that the relation between temperature and metabolic rate was very different for various species of reptiles.

In addition to the direct influence of temperature on metabolism, a seasonal variation has been observed by some workers when determining the oxygen consumption of a particular species at the same temperature during various months of the year. While working on teleost fish, Wells (1935) found quite different temperature metabolism curves for winter and summer animals, depending on their acclimatization. Haugaard and Irving (1943) found that the oxygen consumption of the common cunner, *Tautogolabrus adspersus*, was slightly higher during the winter months than in summer when the temperature was below 15° C. In some amphibians, Noble (1931) stated that a seasonal difference in oxygen consumption could be noted; this difference does not necessarily come at the height of summer but may appear during the mating season.

Much of the earliest investigations on amphibian respiration was reported by Vernon (1897) and Krogh (1916). However, few studies on the respiration of salamanders have been reported in the literature. The rate of oxygen consumption in five species of *Amblystoma* larvae was measured by Helff (1927). Wilder (1937) measured the oxygen consumption of several species in her work on the correlation of various methods of measuring oxygen consumption. Later, Evans (1939), using the methods of Wilder, studied more extensively the oxygen consumption of certain species of Plethodontid salamanders. She found distinct species differences which could be correlated

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with the activity of the organisms. The respiration of *Triturus viridescens* was investigated by Pomerat and Zarrow (1937) at temperatures ranging from 6° to 28° C.

The present investigation of the oxygen consumption of two species of salamanders, the red-backed salamander, *Plethodon cinereus cinereus* (Green), and the two-lined salamander, *Eurycea bislineata bislineata* (Green), was undertaken for the following reasons: (1) to determine any seasonal variation in the oxygen consumption of these salamanders and (2) to study and compare the response of these two forms as measured by oxygen consumption at different temperature levels at various seasons of the year.

MATERIAL AND METHODS

The oxygen consumption of *E. bislineata* and *P. cinereus* was determined at 10° C. and at 1° C. during the three following periods of the year: October–November, February–March, and May–June. Animals tested during February–March were kept in the laboratory from early November in a good nutritional state, whereas animals used during the other two intervals were maintained in the laboratory for a period not exceeding 2 weeks or less than 1 week before beginning the pretesting routine. All specimens used in this experiment were collected in the vicinity of Purdue University.

If the animals were to be tested at 10° C., the following procedure was used during each testing period of the year in order to insure uniform experimental conditions: the animals were fed worms and fruit flies, placed in a constant-temperature chamber maintained at 10° ± 0°.25 C. for 1 week, and then the oxygen consumption was determined.

For the periods of October–November and May–June, when the oxygen consumption was measured at 1° ± 0°.5 C., a longer period of acclimatization to low temperatures was deemed advisable. Animals used for these tests were fed worms and fruit flies and afterward were kept at 10° C. for 1 week. Finally, these individuals were removed to a large constant-temperature room which was set at a temperature of 1° C. The animals were kept in this condition for 1 week before determining their oxygen consumption. The specimens used in the February–March series of experiments were divided into two groups. One group had been in the laboratory at 1° C. without feeding since December. The second group consisted of specimens which were taken directly from outdoor cages at Ross Biological Reserve and had been in a state of hibernation under natural conditions. In this manner, a comparison was possible of laboratory animals and specimens recently recovered from the field.

The oxygen consumption in air of these forms was determined by the closed manometric method described by Wilder (1937). All experiments were conducted under constant-temperature conditions, and the same technique was used for each determination during the various seasons of the year.

Results are reported as cubic millimeters of oxygen consumed per gram of animal per hour.

RESULTS

The data obtained on the oxygen consumption at two temperature levels at different seasons of the year for these two species of salamanders are given in Table 1. Differences were noted in the results obtained at the two temperatures during one particular season and also when oxygen consumption at the same

temperature during different seasons was compared. It can be seen in this table that when tested at 10° C. a steady increase in the rate of oxygen utilization occurred as summer approached. The lowest rate of oxygen consumption was recorded during October–November and the highest for the period of May–June. However, the reverse situation was noted to take place for *P. cinereus* when the experiments were conducted at 1° C. During the period of October–November *P. cinereus* consumed oxygen at a faster rate than it did during February–March; and, in turn, the rate for the latter period was higher than during May–June.

The Q_{10} for *E. bislineata* during October–November was 3.65, for February–March, 2.60, and 2.91 during May–June. Corresponding temperature characteristics of *P. cinereus* for the same three periods were 1.17, 2.16, and 2.63.

Results of the series of tests for February–March conducted at 1° C. showed that specimens of *P. cinereus* immediately taken from outdoor cages had about the same rate of oxygen consumption as animals which were kept in the laboratory for 3 months at lowered temperatures without food. But it was noted that the specimens of *E. bislineata* which had been recovered recently from the field consumed oxygen at a somewhat greater rate than the laboratory animals. This is not considered significant, and the trend, if any, may be correlated with a size difference in the two groups of animals tested, since the field animals weighed less than the laboratory animals.

Dissimilarity in rates of oxygen consumption between different-sized individuals of the same species was observed. When plotting the weight of the animal in grams against the rate of oxygen con-

TABLE 1

SEASONAL OXYGEN CONSUMPTION OF TWO SPECIES OF SALAMANDERS

Species	Months	No. of Tests	No. of Individuals	Average Weight (Gm.)	Average Time (Hr.)	Oxygen Consumed (CuMm/Gm/Hr)
	Determined at 10° C.					
*P.c.c.**	Oct.–Nov.	30	12	0.856	10.8	30.323±1.193
P.c.c.	Feb.–March	25	15	0.835	14.8	33.464±0.72
P.c.c.	May–June	25	25	0.862	13.6	37.432±0.517
E.b.b.†	Oct.–Nov.	25	18	1.117	10.2	38.107±1.109
E.b.b.	Feb.–March	25	13	1.056	11.5	40.208±1.100
E.b.b.	May–June	25	25	1.092	13.6	44.752±0.509
	Determined at 1° C.					
P.c.c.	Oct.–Nov.	25	21	0.695	15.0	18.583±0.902
P.c.c.‡	Feb.–March	34	20	0.592	15.4	15.441±1.082
P.c.c.§	Feb.–March	30	15	0.600	12.6	15.716±1.185
P.c.c.	May–June	25	25	0.844	12.5	14.254±0.570
E.b.b.	Oct.–Nov.	20	20	1.071	17.7	11.740±0.441
E.b.b.‡	Feb.–March	24	15	0.989	15.6	13.683±1.239
E.b.b.§	Feb.–March	12	3	0.789	14.9	16.860±1.358
E.b.b.	May–June	25	25	1.041	12.5	15.345±0.539

* *Plethodon cinereus cinereus.*
† *Eurycea bislineata bislineata.*
‡ Laboratory animals.
§ Field animals.

sumption, it was noted that smaller animals generally consumed more oxygen per unit weight than did larger specimens (Figs. 1 and 2). Although *E. bislineata* had a higher metabolic rate than *P. cinereus* during all seasons when the experiments were conducted at 10° C., this relationship was not evident when tested at 1° C.

DISCUSSION

Many factors influencing the oxygen consumption of animals should be kept in mind when comparing results. Vernon (1897) observed in cold-blooded forms that elevated temperatures caused an increased metabolic rate. This phenomenon was clearly demonstrated in the present paper by comparing results obtained at the two different temperature ranges. Another important factor is that of size differences. It has been well established that smaller animals consume more oxygen per unit of weight than do larger animals. Results obtained when testing animals of varying size within a species is not the same per unit weight and indicate that increasing age and size decrease oxygen consumption. The rate of respiration is more nearly proportional to surface area than to body size.

The nutritional condition of the animal has been demonstrated by various workers to influence directly the rate of oxygen consumption (Smith, 1935). Animals used in the present study were fed in a standard procedure before each test as described earlier, and therefore this factor was not an experimental variable.

In order to ascertain if any physiological difference, as measured by oxygen consumption, existed between animals kept in the laboratory during the winter at reduced temperature and animals recovered from the field in the winter months, a series of determinations were made on two such groups of animals. No significant difference was noticed, and it would seem that acclimatization to laboratory conditions had not resulted. One should keep in mind that this comparative experiment was conducted at 1° C. and that the animals were extremely inactive. At higher temperatures acclimatization might have taken place more noticeably.

A distinct seasonal variation in oxygen consumption was observed in both species of salamanders. As mentioned earlier, other workers have noted seasonal variation in rate of oxygen consumption in various groups of animals, but a similar phenomenon had not been demonstrated previously for salamanders. In both species a seasonal difference could be noticed when determinations were made at 10° C.; an increased rate was observed to be correlated with the approach of the warmer months of the year. This finding is in accord with field observations which show that these animals are more active during spring than in the fall. However, the two species responded differently when the oxygen consumption was measured at 1° C. The decrease in oxygen consumption of *P. cinereus* during the February–March period as compared with October–November may be correlated with the influence of inanition on the organism, or size difference; but, if this were true, why should *E. bislineata* not exhibit the same response? It suggests more strongly that an inherent seasonal cycle is operative in the case of each of these two species of salamanders.

The comparing of species is difficult unless comparisons are made between individuals of similar size. The fact that a small animal, even within a species, consumes more oxygen per unit weight than a larger animal has been well substantiated. Helff (1927) found species differences in oxygen consumption of five spe-

cies of *Amblystoma* larvae of the same age and size. Results reported in the present paper clearly show that, when comparing specimens of equal size, *E. bislineata* has a higher metabolic rate than *P. cinereus* during all seasons of the year when determined at 10° C. However, at 1° C. during October–November, similar-sized individuals of both species consumed the same amount of oxygen. This change in relationship of oxygen-consumption curves at the two temperature levels suggests that an adaptative metabolic process had taken place. In other words, *P. cinereus* showed an increased metabolic rate at 1° C. over what was expected. A similar phenomenon was reported by Haugaard and Irving (1943) in the com-

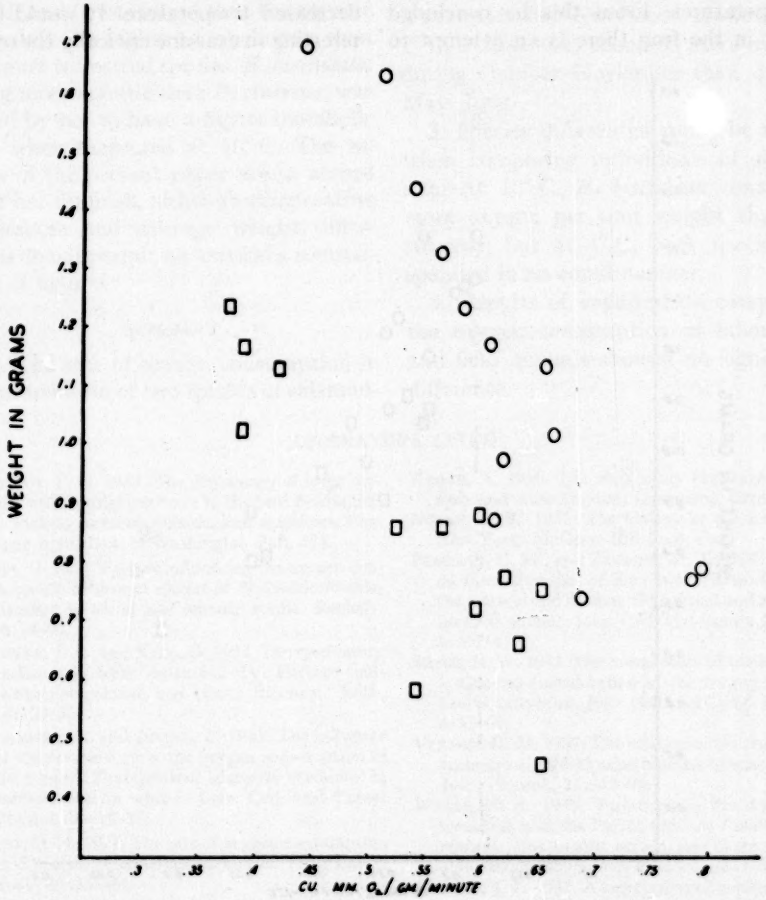

FIG. 1.—The oxygen consumption of two species of salamanders of different sizes measured at 10° C. during October and November.
□ Plethodon cinereus cinereus ○ Eurycea bislineata bislineata

mon cunner. During the winter this species of fish consumed oxygen at a higher rate than in the summer when determined at temperatures below 15° C. However, the difference was too small to enable this animal to remain active during the winter and remain in its summer habitat. Kayser (1940), in studying the heat production of the frog at 0°.5 C. and 5°.0 C., obtained similar values for both temperatures. From this he concluded that in the frog there is an attempt to regulate body temperature at the approach of 0° C. and that these animals do not submit passively to the influence of the ambient temperature. If this explanation is true, one can postulate that, in the case of these two species of salamanders, *P. cinereus* attempts to adjust to the influence of low temperatures by raising its metabolic rate, while *E. bislineata* apparently submits passively to a decreased temperature. It would be interesting to examine critically the oxygen

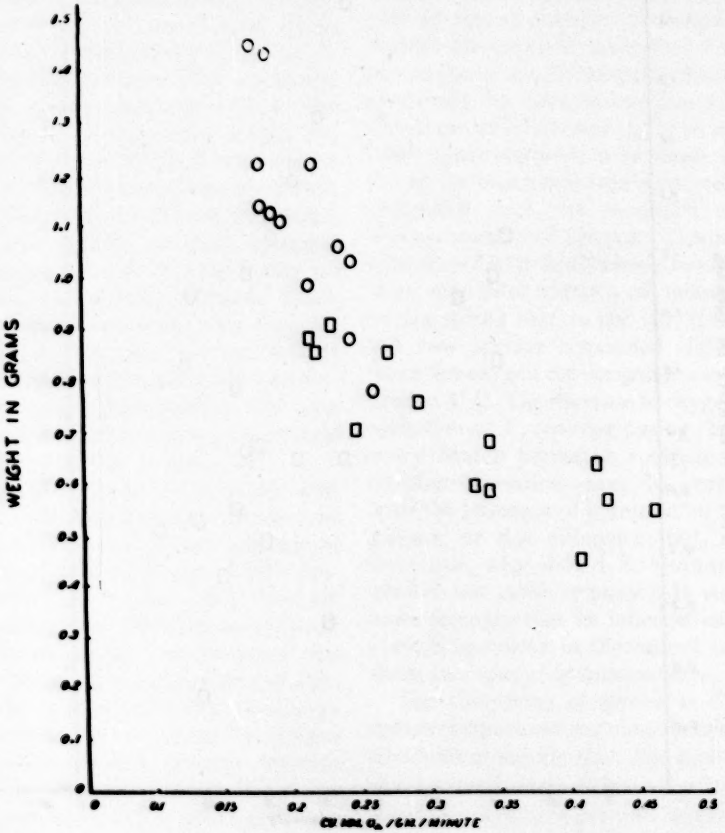

FIG. 2.—The oxygen consumption of two species of salamanders of different sizes measured at 1° C. during October and November.

□ Eurycea bislineata bislineata ○ Plethodon cinereus cinereus

consumption of *E. bislineata* at various low temperatures to determine if it too shows a similar adjustment to cold as does *P. cinereus* but at a different temperature level.

Correlation between habitat and metabolism of Plethodontid salamanders has been suggested by Evans (1939). In her paper the rate of oxygen consumption of four species of salamanders was compared, and it was found that the aquatic animals had a higher metabolic rate than the more terrestrial species. *E. bislineata*, being more aquatic than *P. cinereus*, was found by her to have a higher metabolic rate when measured at 16° C. The results of the present paper are in accord with her findings, although temperature differences and average weight differences do not permit too critical a comparison of figures.

SUMMARY

1. The rate of oxygen consumption in an air medium of two species of salamanders, *Plethodon cinereus cinereus* (Green) and *Eurycea bislineata bislineata* (Green), was studied at two different temperature levels during various seasons of the year by means of a closed manometer system.

2. A seasonal variation in oxygen consumption could be noted for both species. When determinations were made at 10° C., the highest metabolic rate of both species occurred during May–June; the lowest, October–November. However, at 1° C. *P. cinereus* consumed more oxygen during October–November than during May–June.

3. Species differences could be noted when comparing individuals of similar size. At 10° C. *E. bislineata* consumed more oxygen per unit weight than *P. cinereus*, but at 1° C. both species responded in an equal manner.

4. Results of experiments comparing the oxygen consumption of laboratory and field animals showed no significant difference.

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COMPOSITION OF THE PROTEIN MATERIAL IN THE EXUVIAE OF THE MORMON CRICKET ANABRUS SIMPLEX HALD.[1]

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THE insect exoskeleton, because of its importance in conditioning insect behavior, has been a subject of considerable study. In general, these studies have been approached from two points of view—the physical characteristics and the chemical composition of the exoskeletal materials. Early investigations, following the discovery of chitin in the elytra of beetles by Odier (1823), were mainly concerned with this substance. More recently, however, the protein constituents have received considerable attention and are now being regarded as the more important component in the interpretation of cuticular properties.

An unpredictable variability of the chitinous constituent in exoskeletal materials has been observed, not only between species, but throughout the different stages of development within the same species. Fraenkel and Rudall (1940) reported chitin values as high as 60 per cent for the larval cuticle of *Sarcophaga*, while Pepper and Hastings (1943) reported values as low as 1.5–2.5 per cent for the soft body cuticle of *Loxostege* larvae. Anderson and Richards (1942) found that no chitin was present in the iridescent blue scales of the butterfly *Morpho cypris*. Richards (1947) reported that in certain forms of lepidopterous scales consistently negative tests for chitin were obtained, and Picken (1949) points out that when the scales of *Ephestia* are first laid down chitin is absent, this material being incorporated progressively as the scales mature.

Richards (1947) considered protein to be the fundamental constituent of the insect cuticle, while Fraenkel and Rudall (1947) suggest that the basis of the arthropod cuticle structure consists of alternating monolayers of chitin and protein. The fact that no biological membrane, including the insect cuticle, has been studied which is completely lacking in protein may be cited as evidence for the importance of this component in membrane physiology. Present knowledge reveals that the exoskeleton is a complex structure and that information concerning its fundamental constituents is necessary for an understanding of its complicated behavior.

Generally speaking, the cuticle is considered to be divided into two main divisions: the epicuticle and the endocuticle. These are differentiated on the basis of solubility in concentrated hydrochloric acid and on absorption of Mallory's triple stain. The above two layers have been further subdivided into several regions depending upon the species of insect. Richards and Anderson (1942) point out that in the case of the cockroach, *Periplaneta americana*, the epicuticle consists of two layers. Dennell (1946) found the same to hold true for

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Sarcophaga falculata, while Wigglesworth (1945) states that in *Rhodnius prolixus* the epicuticle is composed of four layers. Unlike the epicuticle, the endocuticle is considered by most authors to be composed of only two layers, irrespective of species. In general, the epicuticle is composed of lipoids and proteins, while the endocuticle is composed of proteins and chitin.

Studies on the manner of deposition and the hardening of the cuticle have provided some information concerning its composition. Pryor (1940), working on the oöthecae of the cockroach, showed that it was not digested by pepsin or trypsin nor was it soluble in dilute acids or alkalis. He indicated that it gave a typical Mellon reaction and, further, that, if freshly darkened oöthecae are first dissolved in strong caustic soda solution, they give a positive reaction with ninhydrin and a positive biuret reaction. Both of the latter reactions are very strong in the newly secreted undarkened oöthecae but decrease in intensity as the oöthecae age. The argentaffin test was positive, and, according to both Pryor (1940) and Lison (1936), the biological compounds which will give this test are restricted to polyphenols and polyamines. Pryor (1940) further states that the proteins of the oöthecae and the insect cuticle, in general, have a very low sulfur content. He found that the oöthecae contained 0.15 per cent sulfur, equivalent to 0.56 per cent cystine; and he also found that *Calliphora* cuticle, similarly treated, yielded no sulfur, whereas Trim (1941) found that *Calliphora* larval cuticle contained 0.40 per cent sulfur.

Trim (1941) reported total nitrogen values ranging from 9.0 per cent for the larval cuticle of *Sarcophaga* to 11.8 per cent for the locust abdominal cuticle. Fraenkel and Rudall (1940) reported 9.8 per cent nitrogen in the larval cuticle and 10.9 per cent nitrogen in the puparia of *Sarchophaga*. After autoclaving these cuticles at 135° C. for 5 hours, they found a nitrogen content of 15.2 per cent for the water-soluble fraction of the larval cuticles and 15.1 per cent for the puparia. They also stated that the *Sarcophaga* larval cuticle had a tyrosine content of 2.38 per cent, whereas the puparium contained 1.9 per cent. However, when the increased weight of the puparium is taken into account, it was shown that the total tyrosine content of the two stages is approximately the same.

Fraenkel and Rudall (1947), again working with *Sarcophaga* larval cuticle, recorded a calculated tyrosine content of 2.38 per cent. This corresponds exactly to the value reported in their earlier studies. They further secured a calculated tyrosine content of 2.23 per cent for the puparium, which is significantly higher than the determined value of 1.92 per cent reported in the earlier work. These authors suggested that, during the reaction of the hardening agents with the cuticle protein, the tyrosine residues were altered in a way that prevented their subsequent estimation. They also state that some loss of tyrosine may occur, owing to the drastic treatment of the puparia before the colorimetric estimation of tyrosine by Lugg's method can be carried out.

Trim (1941) reported the water-soluble larval cuticular protein of *Sarcophaga* to contain 5.2 per cent tyrosine, 1.4 per cent tryptophane, and 0.67 per cent glycine. Species variations in the proportions of these proteins in insect cuticle is evidenced by the fact that Trim also found, for the larval cuticle of a Sphinx moth, 11.7 per cent tyrosine, 0.5 per cent tryptophane, and 1.32 per cent glycine. He also noted a decrease of 1.5 per cent

in the tyrosine content of *Sarcophaga* on pupation. He states that a comparison of the prepupae and pupae from the same brood shows that there is a decrease in the total tyrosine in the whole organism of from 1.70 to 0.87 per cent. He further indicated that part of the lost tyrosine, a portion of which came from the cuticle, had apparently been oxidized to form pigmented products.

In studying the water-extractable protein, Trim (1941) points out that this protein, while similar in various insects, is distinctly different from all other proteins with which it has been compared. It differs from gelatin in its high tyrosine and low glycine content as well as in certain physical properties. It is similar to sericin in its amine-N, basic N, nonamino-N, tyrosine, and tryptophane content. It is also similar in that neither one contains any cystine or methionine. The principal difference between the two is in the high serine content of sericin.

These water-extractable proteins apparently undergo changes in the puparium by becoming hard and water insoluble. Pryor (1940) suggested that this protein is tanned by a phenolic substance and that this reaction apparently forms an insoluble complex. However, Fraenkel and Rudall (1940) have shown that this complex can be separated effectively from the chitin by treating the puparia with a hot 5 per cent solution of potassium hydroxide or 5 per cent hydrochloric acid for 1–2 days.

Since all the water-soluble protein fractions obtained from the cuticles of various species of insects had certain characteristic properties, Fraenkel and Rudall (1947) proposed the name "arthropodin" for this material.

Because of the interest in cuticular proteins, it was thought that an investigation of this material in the exuviae of the mormon cricket, *Anabrus simplex* Hald., might yield some interesting information. When mormon crickets occur in outbreak numbers, they offer a ready source for the collection of large quantities of cast skins. While these cast skins or exuviae do not represent the entire exoskeletal system, they do contain the epicuticle and a portion of the endocuticle. It is assumed by many authors that the major portion of the exoskeletal proteins are present in the endocuticle, which in some insects is, at least in part, digested and reabsorbed during the molting process. According to Wigglesworth (1933), the epicuticle of *Rhodnius* is in no way affected by the molting fluid prior to the molt. He states that the endocuticular components of chitin and protein are dissolved and a portion of them reabsorbed. The amount of old cuticle that is dissolved varies considerably between species (see Richards, 1951).

In view of the general lack of knowledge which exists concerning the insect exoskeletal proteins the following investigation was undertaken.

EXPERIMENTAL

The cast skins were collected, washed, and dried in an oven at 100° C. to constant weight. The ether-soluble constituents were then removed by use of diethyl ether in a soxhlet extraction apparatus. The chitin was determined according to the method of Bergmann (1938). The total ash was obtained after incineration in a muffle furnace for 4 hours at 650° C. The Kjeldahl method was used in determining the total nitrogen content of both the exuviae and the chitin. The results of these various analyses were: nitrogen 9.8 per cent, chitin 14.25 per cent, ether extractables 4.47 per cent, and ash 8.72 per cent. The chitin obtained contained 5.4 per cent nitrogen.

Microbiological assay methods were employed in determining the individual amino acids present. In order to check the accuracy of the individual assays, the amino acid content of purified casein was determined concurrently with each amino acid estimation of the exoskeletal material. The procedures employed are as follows:

Organisms.—Cultures used were *Streptococcus lactis* R (ATCC No. 8043), *Lactobacillus arabinosus* 17-5 (ATCC No. 8014), *Leuconostoc citrovorum* (ATCC No. 8081), and *Leuconostoc mesenteroides* P-60 (ATCC No. 8042).

Stock cultures.—Stock cultures were carried using the complete basal medium I of Henderson and Snell (1948) but omitting 50 per cent of the glucose and the sodium citrate and adding 0.5 per cent Bactotryptone, 0.5 per cent Bacto-yeast extract, and 1.5 per cent Bacto-agar. The stock medium for *Leuconostoc citrovorum* included, in addition to the above, 0.4 ml. of Lilly reticulogen per 500 ml. of medium. Stock cultures which were transferred monthly were incubated for 24 hours at 35° C., then stored at approximately 4.5° C.

Liquid medium.—The liquid medium for the growing of the inocula was identical with the stock medium except that the agar was omitted.

Preparation of inoculum.—Transfers were made into the liquid medium from stab cultures. After incubation for 16 hours at 35° C., the cells were centrifuged, washed twice with sterile 0.9 per cent saline solution, and resuspended in sterile 0.9 per cent saline solution approximately ten times the volume of the original liquid medium. One drop of this suspension (approximately 0.05 ml.) was dispensed from a syringe to inoculate each assay tube.

PREPARATION OF HYDROLYSATES

Acid hydrolysate.—The samples (2.5 gm. casein; 4.0 gm. exuviae) were mixed with 50 ml. 3 *N* hydrochloric acid in a 125-ml. Erlenmeyer flask, covered with an inverted beaker, and autoclaved at 15 pounds pressure (121°.5 C.) for 8 hours After the excess hydrochloric acid had been removed by vacuum distillation, the hydrolysates were adjusted to pH 6.8 with sodium hydroxide and diluted with distilled water to 500 ml. final volume. Appropriate dilutions of these solutions were used in the assay tubes.

Alkaline hydrolysates.—(1) Sodium hydroxide with cysteine as the stabilizing agent as described by Kuiken, Lyman, and Hale (1947) was used. The solutions were adjusted to pH 6.8 with sulfuric acid, clarified by centrifugation, and diluted to 500 ml. final volume with distilled water. (2) The samples were mixed with 5 *N* barium hydroxide and autoclaved at 15 pounds pressure for 8 hours. The solutions were heated to 50°–60° C. and adjusted to pH 4.0 with sulfuric acid. After the precipitated barium sulfate had been separated by centrifugation, the solutions were readjusted to pH 6.8 with sodium hydroxide and diluted to a final volume of 500 ml. as before. All hydrolysates were prepared in duplicate.

Basal medium.—Basal medium was prepared in twice the concentration desired, omitting the amino acid to be assayed. The medium used in the majority of the determinations was that of Henderson and Snell (1948). Sauberlich and Baumann's medium (1946–48) was employed for assays with *Leuconostoc citrovorum*. Pyridoxamine and pyridoxal, Sauberlich and Baumann (1949), were omitted from the basal medium for the alanine assay; aspartic acid was omitted from the basal medium for the glutamic acid assay.

Assay procedure.—When desirable, the amino acids were assayed with two different organisms. In general, the results with two assay organisms agreed well. In the case of methionine, *L. citrovorum* gave results approximately 20 per cent lower than did *L. mesenteroides* P-60. This is in keeping with the findings of Steele *et al.* (1949).

The assays were carried out in 19 × 150-mm. test tubes which were held in groups of forty in wire racks. The tubes were not plugged in the conventional manner, but a "lid" arrangement consisting of a metal cover which extended over the top and well down over the sides of the tubes was employed. The top of the metal cover was padded with nonabsorbent cotton wrapped with cheesecloth. This arrangement proved quite satisfactory and time-saving. Three sterility controls included in each rack of tubes verified the reliability of the technique throughout the assay series.

Each tube contained 5 ml. of basal medium, the appropriate amount of standard or test solution, and distilled water to make a total volume of 10 ml. Four test levels of each hydrolysate were run in triplicate. The solutions in each tube were mixed thoroughly. With the metal covers in place, the assay tubes were autoclaved at 15 pounds pressure for 12 minutes. The tubes were cooled, inoculated as previously described, and incubated at 35° C. Incubation periods were 72 hours for all assays except the glutamic acid and alanine assays, which were incubated for 96 hours. The contents of each tube were then titrated electrometrically to pH 7.0 with 0.1 *N* NaOH.

The ml. 0.1 *N* acid produced in the standard solutions were then plotted against the micrograms of amino acid per tube to give a standard curve. From this curve, the concentrations of test solutions were determined in micrograms per milliliter for the original dilutions of the hydrolysate. The results for the assay of each amino acid were then calculated in terms of percentage of amino acid by weight and percentage of the total nitrogen contributed by that particular amino acid. Results of these assays are tabulated in Table 1. For purposes of comparison the values obtained with purified casein and those values for casein reported in the literature are tabulated in Table 2.

Frequent references are made in the literature to the destruction of amino acids during the hydrolysis of proteins particularly in the presence of carbohydrates (see Lugg, 1933; Nicolet, Shinn, and Saidel, 1942; and Borchers, Totter, and Berg, 1942). In order to check this destruction, a mixture containing 3.03 gm. casein and 0.97 gm. starch were hydrolyzed with HCl in the manner previously described. This hydrolysate was assayed for histidine, methionine, and threonine. The results are summarized in Table 3.

The data presented on the three amino acids show that the destruction during hydrolysis of casein in the presence of a carbohydrate ranged from 14.2 per cent for methionine to 2.1 per cent for threonine. The added starch in the above experiment was approximately equivalent to the chitin residue present in the exuviae.

Several efforts were made to secure a satisfactory assay for the amino acid, serine. Since no reproducible and reliable results could be obtained by microbiological assay methods, the determination of this amino acid was reluctantly abandoned. The total sulfur content, as determined by oxidation in a Parr bomb and precipitation as barium sulfate, was

found to be 0.59 per cent. Of this amount, 0.05 per cent was contributed by methionine. Assuming that the remaining sulfur was present as cystine, this amino acid would account for 2.0 per cent of the exuviae. If cystine were present in this amount, it would account for 2.3 per cent of the total nitrogen.

DISCUSSION

On the basis of the amino acid assays listed in Table 1, 70.3 per cent of the total nitrogen in the exuviae was accounted for. The calculated cystine content of 2.0 per cent would account for 2.3 per cent additional nitrogen, thus making a total of 72.6 per cent of the total nitrogen.

Assuming that the serine and other amino acids which may be present and the losses during hydrolysis amounted to 7.5 per cent of the total nitrogen, then the nitrogen accounted for from protein sources would be 80.1 per cent of the total. Since the ether extracted exuvial material contained 10.26 per cent nitrogen, then 80.1 per cent of this, or 8.2 per cent, is the percentage of nitrogen accounted for as protein nitrogen in the ether extracted exuviae. Multiplying this percentage by the usual factor for the conversion of percentage nitrogen to percentage protein (6.25×8.2), a value of 49.1 per cent protein is obtained.

For the purposes of this discussion, it seems reasonable to assume that approximately half of the exuviae is protein. By recalculating the results from Table 1 on

TABLE 1

AMINO ACID COMPOSITION OF ETHER EXTRACTED *Anabrus simplex* EXUVIAE

Amino acid	Assay Organism Used									
	Leuconostoc mesenteroides P-60		*Leuconostoc citrovorum* 8081		*Streptococcus lactis* R		*Lactobacillus arabinosus* 17-5		*Lactobacillus brevis*	
	Per Cent of Total Nitrogen	Per Cent by Weight	Per Cent of Total Nitrogen	Per Cent by Weight	Per Cent of Total Nitrogen	Per Cent by Weight	Per Cent of Total Nitrogen	Per Cent by Weight	Per Cent of Total Nitrogen	Per Cent by Weight
Alanine			12.1	7.9						
Arginine					6.8	2.2				
Aspartic	3.9	3.8								
Glutamic			3.2	3.4						
Glycine									9.3	5.1
Histidine	3.5	1.3								
Isoleucine	2.1	2.0			2.1	2.0				
Leucine	3.5	3.4					3.6	3.5		
Lysine	3.8	2.0								
Methionine	0.22	0.24								
Phenyl alanine	1.3	1.6								
Proline									7.6	6.4
Threonine	2.9	2.5			2.9	2.5				
Tryptophane					0.31*	0.24*	0.30*	0.23*		
Tyrosine	4.6†	6.1†								
Valine	4.8	4.1								

* Hydrolysis with sodium hydroxide stabilized with cysteine.

† Hydrolysis with barium hydroxide.

the basis of a pure protein amounting to approximately 50 per cent of the ether extracted exuviae, the results listed in Table 4 are obtained.

It will be observed that this protein resembles gelatin in that both are high in proline, alanine, and glycine and low in tryptophane and methionine. One striking difference, however, between this protein material and gelatin lies in the tyrosine content. The tyrosine content of gelatin amounts to 0.2 per cent, while the tyrosine content of the exuvial protein material amounts to 12.2 per cent.

The exuvial protein material also resembles silk fibroin, which is likewise

TABLE 2

AMINO ACID COMPOSITION OF CASEIN AND COMPARATIVE LITERATURE VALUES*

Amino acid	Essay Organism Used					Literary Values		
	Leuconostoc mesenteroides R-60	*Leuconostoc citrovorum* 8081	*Streptococcus lactis* R	*Lactobacillus arabinosus* 17-5	*Lactobacillus brevis*	Block and Bolling (1945)	Block and Mitchell (1946)	Steele *et al.* (1949)
Alanine	.....	3.7	.....	.....	.....	2.0—5.7	.....	3.7
Arginine	.....	.....	4.2	.....	.....	4.1±0.2	4.2	4.14, 3.68
Aspartic	7.2	.....	.....	.....	.....	6.3	.....	7.7
Glutamic	.....	22.8	.....	.....	.....	22.8	.....	23.3, 22.8
Glycine	.....	.....	.....	.....	1.2	.....	.....	.....
Histidine	3.4	3.3	.....	.....	.....	2.5±0.3	3.0	3.37, 3.34
Isoleucine	6.1	6.0	.....	.....	.....	6.5	6.5	6.6, 6.6
Leucine	10.1	.....	.....	10.5	.....	12.1	9.9	10.5
Lysine	11.0	.....	.....	.....	.....	6.9±0.7	7.9	8.6
Methionine	3.5	.....	.....	.....	.....	3.5±0.3	3.5	3.3, 2.9
Phenyl alanine	5.7	.....	.....	.....	.....	5.2±0.5	5.6	5.4, 5.3
Proline	.....	.....	.....	.....	11.9	.....	.....	.....
Threonine	4.6	.....	4.7	.....	.....	3.9±0.1	4.1	4.77, 4.6
Tryptophane	.....	.....	1.6†	1.4†	.....	1.8±0.2	1.2	1.39
Tyrosine	5.7‡	.....	.....	.....	.....	6.4±0.4	6.9	6.3, 5.9
Valine	6.2	.....	.....	.....	.....	7.0	6.7	7.6, 7.8

* Percentage by weight of ash-free, water-free casein calculated to 16.0% nitrogen.
† Hydrolysis with sodium hydroxide stabilized with cysteine.
‡ Hydrolysis with barium hydroxide.

TABLE 3

PERCENTAGE AMINO ACID BY WEIGHT IN CASEIN AND IN CASEIN WITH STARCH ADDED

Amino Acid	Organism Used					
	Casein and Starch			Casein Alone		
	Leuconostoc mesenteroides P-60	*Leuconostoc citrovorum* 8081	*Streptococcus lactis* R	*Leuconostoc mesenteroides* P-60	*Leuconostoc citrovorum* 8081	*Streptococcus lactis* R
Histidine	3.3	3.2	.....	3.4	3.3	.....
Methionine	3.0	2.8	.....	3.5	3.0	.....
Threonine	.....	.....	4.7	.....	.....	4.8

high in tyrosine, glycine, and alanine but low in methionine. However, silk fibroin does not contain comparable amounts of proline or the basic amino acids.

No favorable comparison can be made with any of the sclero-proteins such as those obtained from wool, skin, hair, etc.

TABLE 4

CALCULATED COMPOSITION OF EXUVIAL PROTEIN*

Amino Acid	Per Cent
Alanine	15.8
Arginine	4.4
Aspartic acid	7.6
Cystine†	4.6
Glutamic acid	6.8
Glycine	10.2
Histidine	2.6
Isoleucine	4.0
Leucine	7.0
Lysine	4.0
Methionine	0.6
Phenyl alanine	3.2
Proline	12.8
Threonine	5.0
Tyrosine	12.2
Tryptophane	0.5
Valine	8.2

* Based on assumptions explained in text.
† Calculated from sulfur content.

These proteins are all high in cystine, glutamic acid, and arginine, which are found only in moderate quantities in the protein material of the exuviae.

Trim (1941) performed a partial analysis of an exoskeleton protein (arthropodin) and obtained results indicating that this protein had a high tyrosine content but was low in glycine and proline. The results of Trim's analysis do not agree with the values obtained in this investigation. However, the arthropodin investigated by Trim represented the water-soluble fraction from exoskeletal material, while the results obtained in this investigation are based on the total protein material in the exuviae. It is altogether possible that, had the exuvial protein material been fractionated, several fractions with varying amino acid composition may have been obtained, depending on the methods employed in the fractionation.

The chitin was found to contain 0.76 per cent of the total nitrogen present in the exuviae. After deducting the nitrogen from the protein and chitin sources, there remains approximately 20 per cent of the total nitrogen unaccounted for. It is possible that this nitrogen may be involved in polyamine structures produced during the tanning or hardening process of the cuticle, which on hydrolysis would yield simple compounds which are not amino acids.

SUMMARY

1. The exuvium of the mormon cricket, *Anabrus simplex* Hald., has been assayed for its amino acid content by the microbiological assay method. Sixteen constituent amino acids were determined by this method. In addition, the amount of cystine was calculated from the sulfur content, making a total of seventeen different amino acids reported.

2. The results obtained indicate that the protein of the exuviae in some respects resembles gelatin and silk fibroin.

3. Several differences between these proteins and the exuvial protein material have been noted. Based on the analysis reported in this investigation, approximately half of the mormon cricket exuvium is a protein material.

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THE NITROGEN TO PROTEIN CONVERSION FACTOR FOR PUPAE OF THE MOTH, TELEA POLYPHEMUS CRAMER[1]

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For over seventy-five years biochemists have multiplied the weight of the nitrogen present in a protein solution by 6.25 to obtain the weight of the protein present in the solution. The use of the figure 6.25 (called the "protein conversion factor") is based on the assumption that protein contains 16 per cent nitrogen by weight. The actual source of this factor, according to Hiller, Plazin, and Van Slyke (1948), is obscure, for there is no published work which claims its origin. The per cent nitrogen, as has long been known, varies with each type of protein or protein group. Bierry and Vivario (1923) determined the total protein in human plasma both by acetone extraction and by direct coagulation. Nitrogen determinations made upon proteins separated by acetone extraction gave values ranging from 15.03 to 15.45 per cent, while those made upon protein obtained by direct coagulation ranged from 13.75 to 14.00 per cent. These authors did not give their method for determining the nitrogen percentages. Block, Darrow, and Cary (1934) obtained nitrogen values of 14.2 per cent for the protein of cow serum; 14.6 per cent for that of dog serum; and 14.7 per cent for that of human serum. However, they did not give the method by which the nitrogen was determined. They used the phosphate method of Howe (1923) to determine the total protein. Chibnall, Rees, and Williams (1943) found 15.72–15.75 per cent nitrogen in casein; 16.78–16.82 per cent in carboxy-hemoglobin from horses; and 15.69–15.80 per cent in egg albumin. Hiller, Plazin, and Van Slyke (1948) found the average percentage of nitrogen in protein derived from human blood to be 16.02. These latter authors used the Kjeldahl digestion procedure and both gasometric and ammonia distillation methods for determining nitrogen.

Hawk, Oser, and Summerson (1947, p. 100) give a normal range of from 15 to 19 per cent nitrogen in protein. These authors (pp. 108–9) list the nitrogen percentages of many plant and animal proteins. Only one value—31.5 per cent for salmine (a protamine derived from salmon sperm)—is outside this range.

The actual experimental work in determining nitrogen to protein conversion factors has been done upon proteins of many vertebrate tissues, a few plant tissues, and a few invertebrate tissue products. However, the factor 6.25 has been used even for invertebrate tissues to determine the amount of protein present when the weight of the nitrogen contained in the tissues was known. Needham (1931, p. 342) used it to determine the amount of protein in the silkworm *Bombyx mori*, from the nitro-

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gen values given by Russo (1922), and to calculate the protein contained in the sea urchin *Strongylocentrotus lividus*, from the nitrogen values given by Russo (1926). Heller (1932) determined the weight of the nitrogen in different fractions from pupae of the moth *Deilephila euphorbia*, and then used the factor 6.25 to compute the weight of the protein. Hopkins (1950) also used 6.25 for the conversion of total nitrogen to protein in the cestode *Schistocephalus solidus*. Ludwig (1950*a*) used 6.25 for the conversion of total nitrogen to protein in his studies on the effect of starvation on the metabolism of the grasshopper *Chortophaga viridifasciata*. He obtained results which were too high for the protein content of starved grasshoppers, but he pointed out that one error in this procedure is the assumption that all the nitrogen determined is protein nitrogen. He also (1950*b*) showed that, if only the protein nitrogen is used in the calculation, reasonable protein percentages are obtained for both normal and starved insects, and he suggested that the factor 6.25 may be approximately correct for insect protein. To the writer's knowledge the present work represents the first experimental attempt to determine a protein conversion factor for invertebrate tissues.

MATERIAL AND METHODS

Living pupae of the moth *Telea polyphemus* were left in their cocoons and kept under refrigeration at 7° C. to prevent development. As needed, pupae were removed from their cocoons and immersed in anhydrous ether for a period of 1–2 minutes at room temperature to remove the waxy coating from the cuticle. This procedure was used to hasten desiccation. The pupae were then placed in a vacuum desiccator at room temperature and allowed to desiccate for a period of 3–4 weeks. Control experiments indicate no change in the protein as a result of this treatment. At the end of this period each pupa was individually pulverized in a 50-ml. centrifuge tube.

Protein was extracted according to the method outlined by Hiller, Plazin, and Van Slyke (1948). Two fractions were obtained: fraction A contained pure soluble protein, and fraction B insoluble protein plus exoskeleton. Both were dried to constant weight in an oven at 103°–104° C. Weighed samples of each fraction were then furnaced at 600° C. If any ash was recovered after 4 hours, the original samples were repurified. This procedure was repeated until there was no recoverable ash. The fractions were then stored under vacuum desiccation.

The milligrams of nitrogen contained in weighed amounts of each fraction were determined by the Kjeldahl method, and the amount of ammonia formed was measured by titration with 0.01 N HCl. The ratio, milligrams protein/milligrams nitrogen, is the nitrogen to protein conversion factor.

RESULTS

The values obtained in the nitrogen determinations are listed in Tables 1 and 2. Both the per cent nitrogen and the protein conversion factors are given. Table 1 contains the values determined for pure insect protein, and Table 2 those for insect protein plus exoskeleton. The conversion factor, 6.19, obtained in these experiments on pure protein indicates that the use of 6.25 for the conversion of the weight of protein nitrogen to that of protein for insects may be justifiable. It must be pointed out, however, that these determinations were

made upon insect pupae in about the same stage of development. Values may not be constant throughout the entire pupal stage, since this stage is characterized by the breakdown of tissue

TABLE 1

NITROGEN PERCENTAGES AND CONVERSION FACTORS FOR FRACTION A

Sample No.	Weight (Mg.)	Weight of N (Mg.)	Per Cent N	Conversion Factor
1	44.2	7.14	16.16	6.19
2	14.9	2.42	16.23	6.16
3	19.2	3.10	16.16	6.19
4	10.5	1.69	16.16	6.19
5	13.1	2.12	16.18	6.18
6	8.5	1.37	16.13	6.20
7	20.5	3.33	16.23	6.16
8	6.3	1.02	16.18	6.18
9	25.5	4.10	16.08	6.22
10	11.5	1.86	16.18	6.18
Average			16.16	6.19

TABLE 2

NITROGEN PERCENTAGES AND CONVERSION FACTORS FOR FRACTION B

Sample No.	Weight (Mg.)	Weight of N (Mg.)	Per Cent N	Conversion Factor
1	23.1	3.60	15.58	6.42
2	17.6	2.74	15.58	6.42
3	19.2	2.98	15.50	6.45
4	15.5	2.41	15.65	6.39
5	25.6	3.98	15.55	6.43
6	24.2	3.77	15.58	6.42
7	9.4	1.47	15.58	6.42
Average			15.58	6.42

proteins to simpler compounds and later the building-up of these compounds to the proteins of adult tissues.

The analyses of fractions containing both protein and exoskeleton (Table 2) give conversion factors (average 6.42) which are higher than those obtained for pure protein. This fraction contains chitin, a nitrogenous polysaccharide. Calculations based on the structural formula of the chitin unit give a nitrogen to chitin conversion factor of approximately 6.8. However, the actual factor determined experimentally would be expected to vary with the relative proportion of protein and exoskeleton contained in each sample. No method for completely removing the skeletal elements from the protein has been found, and, until such a procedure is devised, we can do no more than perform analyses on the fraction with its exoskeletal contents.

DISCUSSION

In the present work all samples were kept under vacuum desiccation. Hiller, Plazin, and Van Slyke (1948) objected to this procedure and stated: "Proteins are so hygroscopic that completely dried samples cannot be weighed with any accuracy." The writer believes that a fairly high degree of accuracy can be maintained if the samples are kept under vacuum desiccation and weighed with a balance which is also kept in a comparatively dry atmosphere. This result may be achieved by placing sufficient quantities of a desiccant in the balance case. Hiller, Plazin, and Van Slyke continued: "The most uniform analyses are obtained, according to the writers' experience, when the protein is air dried by equilibration with the moisture of the atmosphere, and the samples are weighed for all analyses, including moisture and ash, during a short time interval and under atmospheric humidity and temperature conditions of equilibration." While it is true that approximately uniform results may be obtained in this manner, the actual per cent of nitrogen in pure protein is not being determined unless corrections are made for moisture and ash. Furthermore, the atmospheric humidity varies greatly

from day to day, thereby introducing another source of error. For this reason, the method used in the present work is believed to give protein/nitrogen ratios which are more accurate than those obtained by the procedure advocated by Hiller, Plazin, and Van Slyke or those of other workers who did not make corrections for the moisture and ash content of their samples.

Kirk (1947) pointed out that if proteins are desiccated at high temperatures, it is difficult to determine when weight is being lost solely by the driving-off of excess water and when it is being lost by protein decomposition. This is true at high temperatures, but it is doubtful whether any decomposition takes place at the temperatures of 103° and 104° C. used in the present study.

SUMMARY

1. Pure protein fractions and fractions containing protein plus exoskeleton of pupae of the moth *T. polyphemus* were quantitatively analyzed for their nitrogen content.

2. The average nitrogen percentage determined for the pure protein fraction was 16.16, and the average protein conversion factor was 6.19. The average nitrogen percentage determined for the protein plus exoskeleton fraction was 15.58, and the average conversion factor was 6.42.

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CHANGES IN THE DISTRIBUTION OF NITROGEN DURING THE EMBRYONIC DEVELOPMENT OF THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN)

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NEEDHAM (1931, 1942) and Brachet (1950) give reviews of the published work on biochemical changes associated with embryonic development. These reviews indicate an extensive study of the energy sources and enzyme systems involved in embryogenesis. Many determinations have been made on gross changes in carbohydrate, fat, and protein contained in the developing embryo, vitelline materials, and egg membranes. Comprehensive studies have also been made on the respiratory metabolism of developing eggs of many invertebrate and vertebrate forms. As a result of these studies, Needham (1942, p. 603) made the generalization that during embryonic development there is a succession of energy sources, with carbohydrate preceding protein, and protein preceding fat. This generalization is based on his own work (Needham, 1926) on the energy sources during development of the avian egg and on many observations of respiratory quotients which are usually near unity early in embryogenesis, soon shifting to 0.7, where they remain during the major part of the embryonic period. However, not all the published work supports this contention. Horowitz (1939), working on the gephyrean worm, *Urechis caupo;* Trowbridge and Bodine (1940), on the grasshopper, *Melanoplus differentialis;* and Gregg and Ballentine (1946), on the frog, *Rana pipiens*, found no change in the total nitrogen content during embryogenesis. Concerning the work of Horowitz, Needham (1942, p. 627) expressed doubt in regard to the stability of the total nitrogen figures and suggested that an intake of nitrogen might compensate for a nitrogen loss. He considered Boell's (1935) study of respiratory quotients in the developing egg of the grasshopper, *M. differentialis*, as evidence for his theory, although Trowbridge and Bodine found no change in the total nitrogen during the embryonic development of this species.

Few attempts have been made to fractionate the nitrogen during embryonic development. Horowitz (1939) determined protein, nonprotein, amino, and peptide nitrogen and studied the changes in these fractions during the development of the egg of *Urechis caupo*. He reported a slight increase in the protein nitrogen amounting to 6.4 per cent. This increase occurred at the expense of the amino and peptide fractions. He explained the very slight increase in protein nitrogen by assuming that there is a continuous breakdown of yolk proteins and their resynthesis into the proteins of the embryo. Gregg and Ballentine (1946) made an extensive study of the nitrogen fractions during the embryonic development of the frog's egg and found that the total nonprotein nitrogen amounted to only 2.5 per cent of total nitrogen at the beginning of development. This fraction decreased slightly during cleavage and

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gastrulation and then increased gradually, reaching 125 per cent of the original amount at the end of the embryonic period. Total extractable protein nitrogen and total ultracentrifugable nitrogen remained nearly constant during embryogenesis.

Rothstein (1952) studied changes in total nitrogen, carbohydrate, and lipid during the embryonic development of the Japanese bettle at 30° C. He found a decrease in glycogen during the first 4 days, followed by a decrease in free lipid during the remainder of the embryonic period. However, total nitrogen values remained unchanged. Because of the constancy of the nitrogen values, it was decided to perform fractionations to determine any changes which may occur in the amount of nitrogen in each fraction during embryogenesis. This study should give information concerning the synthesis of protein by the embryo and should indicate whether protein is utilized as a source of energy.

MATERIAL AND METHODS

The eggs used in these experiments were collected daily and handled according to the procedure outlined by Rothstein (1952). They were incubated at 30° C., and nitrogen fractionations were made for each day of embryonic development.

Nitrogen determinations were made by the Kjeldahl procedure. Each fractionation was carried out on a group of a hundred eggs. The dried eggs were thoroughly powdered in a 15-ml. centrifuge tube. A solution consisting of 1 ml. distilled water, 4.5 ml. absolute ethyl alcohol, and 4.5 ml. absolute ethyl ether was thoroughly mixed with the crushed eggs. The mixture was allowed to stand, with frequent stirring, for 2 hours. Separation was accomplished by centrifugation, and the supernatant liquid, containing the dissolved lipids, decanted into a 100-ml. Kjeldahl digestion flask. The residue was washed with another 10 ml. of the alcohol-ether solution, separated by centrifugation, and the supernatant liquid added to that already present in the digestion flask. The ether and most of the alcohol were evaporated; the remaining material was then diluted with 25 ml. distilled water to prevent the decomposition of the alcohol during the digestion process, and the nitrogen content determined. The nitrogen determined by this procedure is lipid nitrogen (Fraction A).

The residue remaining after lipid removal was extracted with 10 ml. of boiling distilled water. The extract was separated by centrifugation and decanted into another 15-ml. centrifuge tube. This water extraction was then repeated and the extract decanted into a third 15-ml. centrifuge tube. One milliliter of two-thirds normal H_2SO_4 and 1 ml. of 10 per cent sodium tungstate were then added to each tube containing the water extract. The contents of the tubes were stirred and then placed in a water bath maintained at 90° C. for 15 minutes to facilitate the tungstic acid precipitation. The precipitate in each tube was separated by centrifugation, and the supernatant fluids decanted into a 100-ml. digestion flask. The residues were washed with distilled water, separated by centrifugation, and the supernates added to that already present in the digestion flask. The nitrogen content of these supernates was then determined. This procedure gave values for the water-soluble nitrogen not precipitated by tungstic acid (Fraction B).

The tungstic acid precipitates were transferred quantitatively to a 100-ml. digestion flask and their nitrogen determined. The nitrogen in this fraction is called Fraction C.

The residue remaining after the removal of the lipid and water-soluble fractions was then transferred quantitatively to a 100-ml. digestion flask and its nitrogen determined. The nitrogen in this fraction (Fraction D) is called "insoluble nitrogen."

TABLE 1

CHANGES IN THE DISTRIBUTION OF NITROGEN DURING THE EMBRYOGENESIS OF THE JAPANESE BEETLE

Age	No. of Individuals	Nitrogen as Per Cent Total Nitrogen			
		Fraction A	Fraction B	Fraction C	Fraction D
Newly laid	600	1.7	4.0	81.4	12.9
1 day	800	3.8	6.0	71.5	18.6
2 days	600	9.3	9.1	50.5	31.1
3 days	900	11.2	9.3	32.4	47.1
4 days	600	10.7	9.9	30.3	49.1
5 days	900	10.1	9.0	24.5	56.4
6 days	800	9.1	9.1	22.3	59.5
7 days	600	8.5	9.7	10.0	71.8
Larva (newly hatched)	600	7.2	15.3	11.1	66.4

TABLE 2

TRANSFER OF NITROGEN DURING EMBRYOGENESIS. FIGURES EXPRESSED AS PER CENT TOTAL NITROGEN

Stage of Development	Fraction A	Fraction B	Fraction C	Fraction D
Newly laid to 1 day	+2.1	+2.0	− 9.9	+ 5.7
1–2 days	+5.5	+3.1	−21.0	+12.5
2–3 days	+1.9	+0.2	−18.1	+16.0
3–4 days	−0.5	+0.6	− 2.1	+ 2.0
4–5 days	−0.6	−0.9	− 5.8	+ 7.3
5–6 days	−1.0	+0.1	− 2.2	+ 3.1
6–7 days	−0.6	+0.6	−12.3	+12.3
7 days to newly hatched larva	−1.3	+5.6	+ 1.1	− 5.4

RESULTS

Total nitrogen remained constant at 3.95 mg/100 eggs until the time of hatching. There was a slight loss of nitrogen at eclosion, the value decreasing to 3.60 mg/100 larvae. This loss, which amounts to less than 10 per cent of the initial amount of total nitrogen, may be associated with the shedding of the egg chorion.

The changes in the distribution of nitrogen for each day of embryonic development at 30° C. are shown in Tables 1 and 2 and in Figure 1. Each fraction is expressed as per cent total nitrogen. Fractions A and B remain relatively low throughout embryogenesis. Fraction A (lipid nitrogen) increases during the first 4 days of development and then decreases gradually during the remainder of the embryonic period. Fraction B also shows an increase during early embryogenesis but then remains constant until eclosion. This fraction is considerably higher in the newly hatched larva. Fraction C decreases very rapidly during the first 4 days and then more slowly and irregularly until the end of the embryonic period. The change in Fraction D is in the opposite direction from that of Frac-

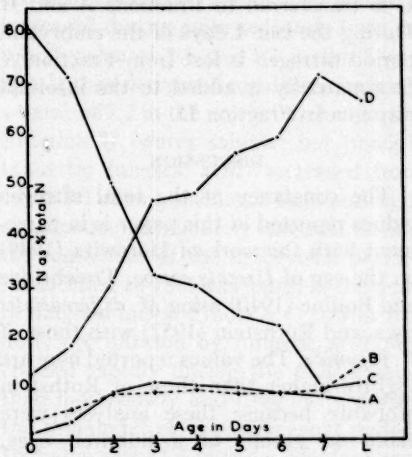

FIG. 1.—Distribution of nitrogen during embryogenesis. *A*, lipid nitrogen; *B*, water-soluble nitrogen, not precipitated by tungstic acid; *C*, water-soluble nitrogen, precipitated by tungstic acid; *D*, water-insoluble nitrogen; *O*, newly laid egg; *L*, newly hatched larva.

tion C, and the curves for these two fractions (Fig. 1) are almost the reciprocal of each other.

The figures listed in Table 2 show that the main shift in nitrogen during embryogenesis is from Fraction C to Fraction D. In addition, during early embryogenesis, a small amount of nitrogen from Fraction C is transferred to Fractions A and B. During the last 4 days of the embryonic period nitrogen is lost from Fraction A. It apparently is added to the insoluble nitrogen in Fraction D.

DISCUSSION

The constancy of the total nitrogen values reported in this paper is in agreement with the work of Horowitz (1939) on the egg of *Urechis caupo*, Trowbridge and Bodine (1940) using *M. differentialis* eggs, and Rothstein (1952) with those of *P. japonica*. The values reported here are slightly higher than those of Rothstein probably because these analyses were made on groups of a hundred eggs, whereas his determinations were made on groups of only fifteen eggs.

To the authors' knowledge, the present work is the first to show very large shifts in nitrogen during embryogenesis. Horowitz (1939) found only a slight increase in protein nitrogen. However, he killed the eggs with trichloracetic acid, an excellent protein-precipitating agent. Hence, his protein nitrogen values are almost the equivalent of the nitrogen contained in Fractions C and D of this paper. When the nitrogen percentages of these two fractions are added together, a loss of 12.5 per cent is demonstrated for the embryonic development of the Japanese beetle. A comparison with the work of Gregg and Ballentine (1946) is not possible, since they used a different fractionation procedure.

The exact nature of the nitrogenous compounds contained in each fraction is not known. The nitrogen in Fraction A (lipid nitrogen) is probably present in phospholipids. Fraction B (water soluble, not precipitated by tungstic acid) may contain amino, peptide, and other nonprotein nitrogenous compounds such as soluble waste products (urea, ammonia, allantoin, etc.). Fraction C (water soluble, precipitated by tungstic acid) probably contains metaprotein, proteose, and peptone portions of the egg. Since boiling water was used in the extraction process, it is likely that the more complex proteins are coagulated and left in the insoluble residue. Fraction D contains the nitrogen of the insoluble portions of the egg membranes, yolk proteins, and proteins of the embryo. The increase in this fraction may indicate the synthesis of structural proteins in the developing embryo. Its rate of increase may be an indication of the rate at which embryonic tissue structures are formed.

There is no evidence that nitrogenous compounds are used as an energy source in the Japanese beetle egg. Therefore, this study fails to substantiate Needham's generalization regarding the succession of energy sources, with carbohydrate preceding protein, and protein preceding fat. Total nitrogen remains constant throughout the entire embryonic period. Furthermore, there appears to be no basis for his suggestion that an intake of nitrogen may balance its utilization. The figures contained in Table 1 present no evidence for the accumulation of waste nitrogen. Fraction B increases during early embryogenesis. However, it remains approximately constant from the second day until the end of the embryonic period. The higher value obtained for this fraction in the newly hatched larva may indicate an accumulation of waste nitrogen produced during the period of greater activity associated with eclosion.

A comparison of these results with those of Rothstein (1952) indicates that a possible shift in the mechanism of development may occur after the fourth day of incubation at 30° C. Rothstein found that, during the first 4 days (newly laid egg to 3-day egg), glycogen decreases and the concentration of reducing substances increases. Since the breakdown of glycogen is greater than is required for the synthesis of reducing compounds, it was suggested that glycogen is used as a source of energy during this period. No decrease of fats occurred until after the fourth day. Hence, Rothstein suggested that, during early embryogenesis, glycogen serves as the main source of energy. This period is also characterized by a very low rate of oxygen consumption (Rothstein, unpublished work). It is the so-called "formative period" of Fink (1925). It is also the time during which lipid nitrogen increases and there is a rapid shift of nitrogen from Fractions C to D. The low rate of oxygen consumption and the utilization of glycogen suggests the possibility that anaerobic glycolysis may be the mechanism for energy production. Rothstein found that following the fourth day (4-day to 7-day egg), the rate of oxygen consumption increases rapidly, and free fats are utilized. Following the fourth day, lipid nitrogen decreases, and the rate at which nitrogen shifts from Fractions C to D is relatively slow and irregular. The high rate of oxygen consumption suggests an energy source involving a different enzyme mechanism, possibly the cytochrome system. A study of these mechanisms in the Japanese beetle egg is now in progress.

SUMMARY

1. Nitrogen fractionations were made on Japanese bettle eggs for each day of development at 30° C.

2. Total nitrogen remained constant at 3.95 mg/100 eggs throughout embryogenesis. On hatching, it decreased to 3.60 mg/100 larvae, probably as a result of the loss of the chorion.

3. The nitrogen values for each fraction, expressed as per cent total nitrogen, are as follows: Fraction A (lipid nitrogen) increased during embryogenesis from an initial value of 1.8 to 11.2 in the 3-day egg. It then decreased regularly, reaching a value of 7.2 in the newly hatched larva. Fraction B (water soluble, not precipitated by tungstic acid) increased from 4.0 to 9.1 during the first 3 days and then remained approximately constant during the remainder of the embryonic period. An increase to 15.3 was found in the newly hatched larva. Fraction C (water soluble, precipitated by tungstic acid) decreased rapidly during the first 4 days, then more slowly and irregularly during the remainder of the embryonic period. Its value was 81.4 in the newly laid egg; just before hatching, it was 10.0 The change in Fraction D (water insoluble) was opposite in direction from that of Fraction C. Its initial value was 12.9; just before hatching, it reached a high value of 71.8. A loss to 66.4 (probably associated with the loss of the chorion) occurred at eclosion.

4. The constancy of the total nitrogen values and the absence of any evidence for the accumulation of waste nitrogen are considered as an indication that Needham's generalization regarding the sequence of energy sources during embryogenesis may not apply to the Japanese beetle.

5. It is suggested that a possible change in the mechanisms involved in embryogenesis may occur after the fourth day of embryonic development.

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THE EFFECTS OF TOPICAL APPLICATIONS OF CHEMICAL AGENTS ON HAIR DEVELOPMENT[1, 2]

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RELATIVELY little work has been done on the effects of topical applications of chemical agents on hair development. Butcher (1940) finds that external applications of cantharides, capsicum, xylene, and benzoic acid stimulate hair growth. Simpson and Cramer (1943) produce marked alterations of the integument, including epilation and hyperplasia of the epidermis, by treatment with 20-methylcholanthrene. There is, however, little relating the stage of hair development at the time of treatment to the effect observed. For this study various chemical agents are applied at known stages of the developmental cycle, in order to test their effects on the initiation, acceleration, or retardation of hair development.

MATERIAL AND METHODS

Three chemical agents were selected for this study: benzene, ether, and a concentrated aqueous solution of K_2HPO_4. Eighteen mice of the C57 Black strain were prepared in the following manner: Four areas were selected along the back, two on either side of the mid-line. The two anterior regions were clipped and the two posterior ones plucked. Since David (1934) has shown that plucking initiates the development of the next hair generation while clipping is completely ineffective in this respect, it is possible to test the ability of these agents to retard or accelerate hair formation in the active previously plucked regions and to induce development in the inactive clipped areas. Each chemical was applied to the areas on the left sides of six of the animals, while the right sides remained un-

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painted to serve as control. The applications, begun 6 days after plucking, were made daily for 6 days. Biopsies were taken on the fifth day prior to painting, and again on the eighth, twelfth, and sixteenth days after the beginning of the painting. Gross observations were made throughout.

RESULTS

K_2HPO_4

At the time of the first application of the phosphate solution, the skin of the plucked area was darkening, owing to the appearance of pigment in the bulbs of the hair follicles (Chase *et al.*, 1951). On the fifth day of treatment the first hairs emerged through the skin and elongated at the normal rate, reaching full length in about 8 days (19 days after plucking). In the untreated control region, development proceeded in a similar manner. Histological examination of the plucked area after treatment revealed no apparent differences between sections of treated and untreated skin. In both, development proceeded in a normal manner.

In the unplucked region at the time of the first application, the skin showed the normal white color indicative of resting follicles. There was no change in this appearance during the first 3 or 4 days of painting, but on the last days slight epilation was observed. Hair loss continued, so that by the sixth day after the last painting, the region was sparsely covered and revealed dark skin. New hairs emerged 2 or 3 days later and lengthened at the normal daily rate, reaching completion in 9 more days (24 days after the initial application). Throughout this period, the unpainted control region remained unchanged. Tissues taken from the painted unplucked region after four applications revealed follicles which appeared to be in 1-day anagen; tissues taken 3 and 8 days later revealed follicles in 4-day and 8-day anagen, respectively. It should be noted that if development commenced at the time of the fourth application, as indicated by the appearance of the 1-day anagen stage at that time, development from then on proceeded at a normal rate.

ETHER

On the day of the first application of ether, the skin of the plucked area was faintly dark. The next day the skin was darker, but also inflamed and dry. Before the third treatment, the painted area was drawn and puckered and covered with scabs. On the last day of painting, short hairs were present. In another week (19 days after plucking), the hairs of both the treated and the untreated areas reached completion. Histological examination revealed parallel development in the treated and untreated regions.

In the clipped region at the time of the first treatment, the skin appeared the normal light color. After two applications of ether, the skin became incrusted. By the end of treatment, there was evidence of epilation, which became pronounced in 2 or 3 days. The crusts were large and numerous, and around them were patches of dark skin. Fourteen days after the initial application, the scabs were lost, revealing portions of dark skin. Hairs appeared in the dark patches within a few days. These hairs attained full length at the normal rate. In the first tissues taken, the follicles were in the telogen phase. Seven days later, however, the follicles appeared to be at the 3-day anagen stage. Later biopsies revealed development proceeding at the normal rate.

BENZENE

At the time of the first application of benzene, the skin of the plucked area was

just darkening. After one treatment, the skin became inflamed, dry, and scaly, and after the second it was drawn and partially incrusted. By the end of treatment, most of the scabs were lost, leaving the skin dark and contracted. Seventeen days after plucking, short hairs were observed on the treated side, while those on the control side were close to completion. By the twenty-second day after plucking, however, the hairs of both sides were full length. Microscopic examination of the plucked area after four treatments revealed much damage to the skin. Each of the layers of the epidermis was thickened, particularly the strata corneum and malpighii. The dermis, also thickened, contained many macrophages, and the blood vessels of the dermis and the hypodermis were distended. Hair formation on the treated and control sides proceeded normally. The delay in the emergence of hairs on the painted side was due to the increased thickness of the skin, which caused the bulbs of the follicles to lie farther below the surface. By the twenty-second day, however, there was a decrease in the skin thickness, allowing the formed hair shafts to protrude.

The appearance of the clipped area at the time of the first application was typical of the resting stage. After a single application, the skin became inflamed and dry. By the time of the last painting, this area was covered with scabs, and there was evidence of epilation. After 5 more days, the treated area was completely epilated and the skin dark. Sixteen days after the initial painting, hairs about 2 mm. in length were present. These hairs reached full length about 21 days after the first application. Throughout this period there was no change on the control side. The changes in the skin of the clipped portion paralleled those in the plucked area. At the time of the fifth treatment, the follicles were still in the telogen phase. Three days later, however, there was some downgrowth of the follicle, giving the general appearance of a normal 3-day stage. The dermis and epidermis surrounding the old club appeared hardened and keratinized. Beneath this, there was a new layer of stratum malpighii continuous with the external sheath of the follicle. By the twelfth day the scabs were gone and with them the old hairs and their clubs. The new bulbs were well developed and appeared as normal 7-day stages. Development beyond this proceeded normally.

In general, treatment with these chemical agents during the inactive phase results in the premature initiation of hair development, while treatment during the active phase has no effect on development.

DISCUSSION

Treatment of the clipped area with the phosphate solution results in gradual epilation over a period of 6 days, followed by the appearance of the next hair generation. The commencement of the new development coincides with the time of hair loss. Two interpretations of this are possible: the treatment may act directly to produce epilation, which, in turn, serves to stimulate development in a manner similar to plucking, or it may initiate development directly and effect epilation only incidentally. The evidence here favors the latter view. Since epilation is gradual rather than abrupt, it would be expected, if this were the inducing mechanism, that development would not be initiated simultaneously in all follicles but that variation in stage of development would be observed. Histological examination revealed a general uniformity in stage of development among the follicles. This suggests that epilation is

incidental and that the primary effect of treatment is the initiation of a new hair development.

Treatment with ether or benzene results in severe damage to the integument. This is evidenced by inflammation and thickening of the skin, scab formation, and epilation. In regions in which the follicles were inactive at the time of treatment, these injuries are followed shortly by new hair generations. Epilation after benzene or ether treatment follows the same pattern as that established after phosphate treatment. The same view is held here that epilation is a coincident effect and not directly responsible for stimulating the next hair generation.

Histological examination of the skin reveals distention of the blood vessels of the dermis. Butcher (1940) reports increased vascularization followed by a new hair growth after external applications of various chemical irritants. He attributes the initiation of development directly to the increased blood supply. His observation (Butcher, 1943) that there is increased oxygen consumption in the skin just prior to activity in the hair germ is also suggestive in this respect. It is possible, then, that the augmentation of the blood supply after ether or benzene treatment is responsible for the stimulation of development.

Scab formation as a consequence of treatment precedes hair regeneration and may therefore be involved in its initiation. The scab may include epidermis and portions of the dermis, sebaceous glands, and hair follicles. Prior to the sloughing of the scab, a new basal layer of epidermis appears beneath it. Bishop (1945), in studies of wound healing in human skin, intimates that this new malpighian layer regenerates from the epithelial layers of the underlying hair follicles, as well as from surrounding epidermis. It may be that the proliferation in this external sheath required for the regeneration of the epidermis also stimulates similar proliferative activity in the hair germ (Chase and Montagna, 1951). It is interesting in this respect that hair development commences shortly before the shedding of the scab. This suggests the possibility that the external sheath acts as an intermediary in the normal initiation of development and in the initiation of development following plucking. It has been shown that the earliest proliferation in the hair germ occurs concomitantly with mitotic activity in the external sheath. The external sheath is in more intimate association with the club than is the hair germ. It is possible that the disturbance of the club from plucking stimulates this outer sheath, which then transmits the stimulus to the hair germ. The germ is activated and responds by proliferating, as does the external sheath itself.

Although the mechanisms of action are not clear at this time, certain facts are evident. Treatment is effective only during the telogen phase, and its response is the development of a normal hair. The proper conditions for the succession of normal development must be established, then, during early telogen. Various stimuli, including plucking, chemical agents, and the normal stimulus, are capable of setting this process in motion. After the stimulus is applied, development proceeds normally. In the absence of the proper conditions or of a suitable stimulus, development does not proceed at all. Once under way, its accomplishment cannot be influenced unless treatment is so drastic as to destroy the mechanisms of development. In a later paper the effects of X-irradiation will be reported, in which, following treatment development ceases instead of continuing

abnormally or producing an imperfect product.

It is concluded, then, that the truly critical period with regard to the effects of certain external influences is not the stage of active hair production but rather the developmentally inactive phase. The retardation or acceleration of development which has been reported is not in terms of the rate of hair production so much as in terms of the time of the initiation of production.

SUMMARY

Repeated applications of K_2HPO_4 solution, ether, and benzene were made to regions of the skin in which the hair follicles were active or inactive. None of these agents has an effect on hair development if applied during the active phase. When applied during the inactive phase, the phosphate solution produces epilation and initiates hair regeneration, while the benzene and ether, in addition to these effects, produce inflammation and thickening of the skin and scab formation. In all cases the rate of development and the ultimate product are normal. It appears that the initiation of development is the primary effect of treatment and that epilation is a separate effect. It is suggested that these agents may stimulate the hair germ indirectly through the external sheath. The increase in blood supply, at least for benzene and ether, may also be a factor.

It is concluded that the truly critical stage in the developmental cycle with regard to the effects of certain external influences is not the stage of active hair formation but rather the developmentally inactive phase. The resting period may be shortened, but the rate of growth, once initiated, is not changed.

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GROWTH INHIBITION OF FREE-LIVING PROTOZOA BY ACTIDIONE[1]

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THE antibiotic actidione was isolated by Leach, Ford, and Whiffin (1947) from *Streptomyces griseus*, and its chemical composition was described by Ford and Leach (1948). The spectrum of activity, as determined by Whiffin (1948, 1950), indicated that bacterial growth is relatively unaffected but

[1] Aided by a grant from the Damon Runyon Foundation (DRIR-121).

that yeasts are generally quite sensitive, as are a number of human and plant fungal pathogens. Preliminary assays (Loefer and Matney, 1951) indicated that multiplication of certain free-living protozoa was markedly inhibited by this substance. The results of additional experiments are presented in this report.

MATERIAL AND METHODS

The crystalline actidione used in this investigation was obtained from the Upjohn Company through the courtesy of Dr. Jared H. Ford. The organisms used included a colorless strain of *Euglena gracilis* var. *bacillaris* and thirteen strains of the ciliate *Tetrahymena geleii*,[2] some or all of which were used in previous investigations (Loefer, 1951*a*, 1951*b*, 1952). Unless otherwise stated, all experiments were carried out using a medium containing Bacto Casitone, 2 per cent; Bacto Yeast Extract, 0.25 per cent; and salts in milligram percentage concentrations as follows: NaCl, 20; $CaSO_4$, 5; $MgSO_4$, 2.5; KNO_3, 20; KH_2PO_4, 10; and $FeCl_3$, 0.5. Actidione-containing media was autoclaved, since preliminary tests had shown that there was no appreciable loss of potency. Cultures were established in 22 × 175 mm. Pyrex tubes, each of which contained 10 ml. medium. Inoculations were effected by aseptic transfer of 0.2 ml. of a rapidly multiplying stock. Growth determinations were made after designated periods of incubation at 27°.5 C. Since both comparative counts, made according to the method outlined by Hall, Johnson, and Loefer (1935), and relative optical densities were used as criteria of antibiotic effect, all tubes were selected for uniform diameter and light transmission. Readings were obtained with a Coleman Jr. spectrophotometer at a wave length of 610 mμ.

[2] Dr. John O. Corliss (personal communication) designates *pyriformis* as the type species of the genus *Tetrahymena*.

RESULTS

Serial sets of tubes with actidione concentrations of 10, 5, 1, 0.5, 0.1, and 0 (controls) μgm/ml medium were prepared in triplicate and autoclaved. One set was inoculated with *T. geleii* H, another with strain WB, and the third with *Euglena*. Determinations following the incubation periods indicated are shown in Table 1. Inhibition was complete with

TABLE 1

EFFECT OF ACTIDIONE ON GROWTH. TETRAHYMENA CULTURES INCUBATED 48 HOURS, EUGLENA FOR 8 DAYS; INITIAL pH 5.0

μGM. PER ML. ACTIDIONE	T. GELEII WB		T. GELEII H		E. GRACILIS	
	X*	%†	X*	%†	X*	%†
0 (controls)	111	100	164	100	178	100
0.1	.....	.....	.....	.....	157	88
0.5	97	87	150	91	82	46
1.0	83	75	131	80	55	31
5.0	10	9	15	9	13	7
10.0	1.3‡	.....	1.7‡	.....	2.3‡	.....

* Thousands of organisms per milliliter medium.
† Percentage of control.
‡ No greater than initial count.

10 μgm/ml and more than 90 per cent complete at the 5-gamma level in every case. The data indicate that the inhibition curve for *Euglena* drops more sharply than does that for either strain of *Tetrahymena*.

The effect of hydrogen-ion concentration on activity was determined in a medium containing 5 μgm/ml by comparing growth at various pH levels, as indicated in Table 2. The inhibitory effect was least in the range from pH 7 to 9, with lowest activity evident around pH 8. Inhibition by actidione was markedly greater at higher hydrogen-ion concentrations.

Since the effect was apparently greater at pH 9 than at 8, the question arose whether potency was irreversibly lost in

a neutral or alkaline solution, or whether this change might be correlated with ionization of the molecule. To test this hypothesis a 500 μgm/ml aqueous actidione solution was adjusted to pH 10 with addition of sodium carbonate, and immediately thereafter 0.5 ml. was mixed with 49.5 ml. of medium at pH 5 to give a final concentration of 5 μgm/ml. This medium was tubed and autoclaved. The procedure was repeated after 15 minutes, 1, 3, and 5 hours. The entire series was then inoculated with *T. geleii* WB. After 48 hours incubation, light transmission readings on the cultures were taken and recorded (Fig. 1). It is evident that there was a progressive and permanent loss of activity, as shown by more abundant growth when the exposure time of the actidione sample to the high pH was increased. The decreased growth at pH 9, therefore, reflects the effect of unfavorable pH rather than recovery of actidione activity.

TABLE 2

TETRAHYMENA GELEII, WB. EFFECT OF pH ON ACTIDIONE ACTIVITY

pH		Thousands of ciliates per milliliter	
Initial	At 48 Hr.	24 Hr.	48 Hr.
5.0.....	5.0	*M*	*N*
5.9.....	5.9	*M*	*M*
7.0.....	7.0	62	79
8.0.....	7.9	72	101
9.0.....	8.7	50	58

* *M* = motile; *N* = not viable.

Observations on the growth of thirteen strains of *T. geleii*, in aliquots of a medium containing 5–20 μgm/ml actidione, indicated that there was considerable difference with respect to antibiotic tolerance. After several days of growth the strains were divided into four groups on an empirical basis and designated: (1) very sensitive—GP and Y; (2) average —H, WB, W, WR, E, and EZ; (3) moderately tolerant—EB, L1, L2, and L3; and (4) highly resistant—S.

Using strain WB, an attempt was made to obtain a more tolerant strain by the procedure of successive transfer in a medium containing a high level of actidione. Fourth-passage cultures from media containing 5 μgm. actidione and from similar cultures not grown with actidione were used as stocks for inoculating respective series of tubes containing actidione in micrograms per milliliter as follows: 10, 5, 1, 0.5, 0.1, and 0. After 48 hours incubation, growth determinations were made on all cultures. From these the percentage of growth was computed for cultures at each antibiotic concentration. The comparative values are shown graphically in Figure 2. Actidione tolerance was considerably greater in the strain subcultured with the antibiotic. Actually this strain could withstand as much as 10 μgm., whereas the parent-strain could not.

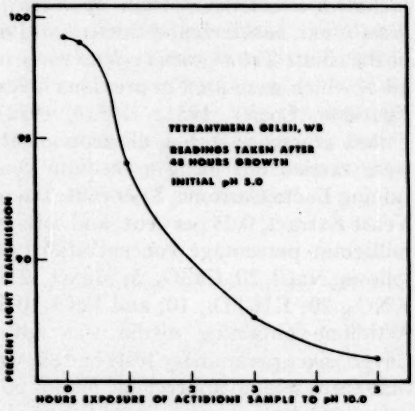

FIG. 1.—Loss of activity of actidione in alkaline solution.

The preliminary observations on strain WB indicated that it might be quite possible to develop a highly tolerant strain by employing a series of step-up transfers, in the course of which cultures displaying good growth in the highest available concentrations of actidione are selected as stocks for inoculation of higher level actidione media. Strain S was selected for this purpose, since it already exhibited the highest resistance to actidione of all strains tested. One per cent glucose was added to the actidione-containing medium to aid in maintaining a low pH, so that full antibiotic potency would be retained over a long period of incubation. This effect is accomplished owing to the formation of acids from the sugar, which offset the effect of alkaline substances formed from other constituents of the medium during growth (Loefer and McDaniel, 1950). By employment of a special procedure, a variant has been obtained which grows well at 100 μgm/ml (Matney, MS).

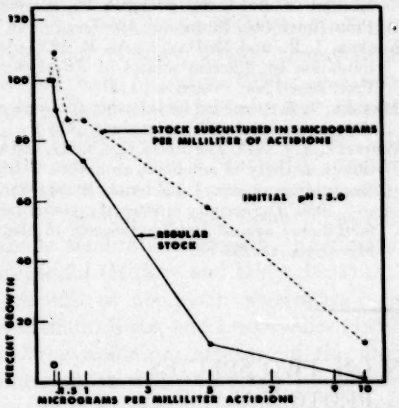

FIG. 2.—Comparative growth of actidione-resistant and normal strains of Tetrahymena geleii, Wb.

Some experiments were also carried out in which the concentrations of Bacto Casitone and Bacto Yeast Extract were varied to determine whether these substances inhibited the activity of actidione, but it appeared that, even if these constituents were reduced by as much as 50 per cent, no relative difference in growth response to actidione was noted.

DISCUSSION

These experiments demonstrate that various strains of *T. geleii* and the flagellate *E. gracilis* are inhibited by concentrations of actidione as low as 1 μgm/ml or less. There are no published reports which indicate that any other antibiotics in concentrations as low as this inhibit growth of these species, although McCowan[3] found that dilutions up to one part in ten million were active *in vitro* against *Endamoeba histolytica*.

On the other hand, most of the antibiotics which were tested on protozoa (cf. Table 3, Blumberg and Loefer, 1952) were relatively not inhibitory unless used in very high concentrations. Preliminary observations on the effect of polymyxin B indicate that its order of effectiveness is intermediate, since it is inhibitory to *T. geleii* in the range of 100–500 μgm/ml under similar conditions.

It appears from these observations that the most potent inhibitors of free-living protozoa are also relatively effective against certain fungi.

SUMMARY

1. Thirteen strains of *Tetrahymena geleii* and *Euglena gracilis* var. *bacillaris* (colorless) were studied with respect to actidione tolerance. Quantities of 1 μgm. per millileter or less, in a medium containing Bacto Casitone and Bacto Yeast Extract at pH 5–6, markedly inhibited growth of all forms.

[3] M. C. McCowan, personal communication.

2. *Euglena, T. geleii* GP and Y did poorly with 5 μgm. per milliliter of the antibiotic.

3. Of the remaining ciliates, strain S was the most highly resistant and was capable of growing at the 20-μgm. level.

4. By employing a special procedure (Matney, MS) a variant of strain S was conditioned to existence in a medium containing 100 μgm. per millileter.

5. At pH levels above 6 actidione potency was lost.

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EFFECT OF NEOMYCIN ON TWO SPECIES OF FREE-LIVING PROTOZOA[1]

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RESULTS of experiments on cultures of isolated strains of nonparasitic protozoa grown in media free of other microörganisms, to test the effect of penicillin, streptomycin, and chloromycetin, have been reported by Loefer (1949, 1951*a*). Preliminary investigations using actidione and bacitracin have also been made (Loefer and Matney, 1951; Loefer, 1952). In the course of assaying neomycin on various organisms, Bennett (1950)[2] discovered that growth of *Euglena gracilis* var. *bacillaris* was blocked. Inasmuch as no other reports were found dealing with the effect of this recently discovered antibiotic on free-living protozoa, experimental work was initiated in order to (1) obtain data on the quantitative changes in cell numbers in cultures of *Euglena*, (2) compare the green and colorless strains of this flagellate, (3) determine to what extent a ciliated protozoan would be inhibited, and (4) discover if changes in cell size would also occur. Preliminary results of these experiments were previously reported (Blumberg and Loefer, 1951).

Neomycin is obtained from the puri-

[1] Aided by a grant from the Damon Runyon Foundation (DRIR-121).

[2] R. E. Bennett in a personal communication and as reported by Felsenfeld, Volini, Ishihara, Bachman, and Young (1950).

fied concentrate of culture medium of certain strains of *Streptomyces fradiae* (*Actinomyces fradii*). It was discovered by Waksman and Lechevalier (1949), and its preparation and properties have been described (Swart, Hutchison, and Waksman, 1949; Waksman, Lechevalier, and Harris, 1949; Waksman, 1950; Waksman, Katz, and Lechevalier, 1950). It is a heterogeneous substance composed of at least three fractions that have been isolated as apparently pure components. These are: neomycin A (Peck, Huffhine, Gale, and Folkers, 1949); neomycin B (Regna and Murphy, 1950); and neomycin C (Dutcher, Hosansky, Donin, and Wintersteiner, 1951). These are quite distinct from a "factor X" named fradicin (Swart, Romano, and Waksman, 1950), formed by the same organisms which elaborate neomycin. Fradicin is fungicidal (Hickey and Hidy, 1951).

A unit of neomycin was defined by Waksman, Katz, and Lechevalier (1950) as "the minimum amount of the antibiotic which will completely inhibit the growth of *Escherichia coli*, A.T.C.C. 9637 in one milliliter of nutrient agar." Bacteriostatic or bactericidal activity was found *in vitro* against many species of gram-positive and gram-negative bacteria, at antibiotic concentrations ranging from 0.006 to more than 33 units per milliliter of culture medium (Hobby, Lenert, and Dougherty, 1949; Felsenfeld, Volini, Ishihara, Bachman, and Young, 1950; Steenken, Wolinsky, and Bolinger, 1950; Poth, Fromm, Wise, and Hsiang, 1950; Clancy, 1951; Kadison, Volini, Hoffman, and Felsenfeld, 1951). Mycobacteria, including some streptomycin-resistant strains of the tubercle bacillus, were especially susceptible in these *in vitro* investigations. Several species of actinomycetes, including *Streptomyces fradiae*, were sensitive to low concentrations. Two strains of Treponemataceae required only 0.15 units per milliliter, but the viruses which were tested *in vitro* were resistant to relatively large amounts. Growth of fifty-three strains of molds and yeasts was uninhibited. *Endamoeba histolytica* was the most sensitive of the parasitic protozoa which were investigated *in vitro*, with complete inhibition occurring at 2.5 to 10 units per milliliter. The flagellate, *Trichomonas vaginalis*, required an average of 100 units per milliliter, while the hemoflagellates (one strain of *Leishmania donovani* and one strain of *Trypanosoma cruzi*) tolerated more than 200 units per milliliter.

MATERIAL AND METHODS

The neomycin[3] used in these experiments had a designated potency of 240 units per milligram. Appropriate amounts were dissolved in aliquots of culture broth,[4] which were then sterilized by passing through ultrafine fritted glass filters. Portions of the aliquots were added aseptically to additional sterile medium in culture tubes to obtain concentrations of 0 (controls), 25, 50, 75, 100, 125, 250, 400, 500, 800, 1,200, 1,600, and 2,000 units per milliliter. Three control cultures, containing one of the protozoan strains being investigated, were set up for each series; there was no neomycin in the medium. Test culures were also established in triplicate for each concentration of neomycin. This procedure was repeated for the other strain being tested in the series. Final volume of each culture was 10 ml., and, as the culture tubes were of approximately the same size, the height of the liquid medium was the same for control and test cultures. Hydrogen-ion

[3] Obtained through the courtesy of Dr. Augustus Gibson, Merck and Company, Rahway, N.J.

[4] An aqueous solution of 2 per cent Bacto Casitone and 0.25 per cent Bacto Yeast Extract.

concentration prior to inoculation was within the range of pH 7.1–7.4.

The methods of inoculation, incubation, and testing for viability were similar to those described previously (Loefer, 1951*a*). A 0.2-ml. inoculum from a stock culture in the logarithmic phase of growth (24–36 hours) was added aseptically to each culture tube. Incubation was at 27°5 C. in a thermostat-controlled water bath. Two protozoan species were used in the experiments: *Tetrahymena geleii*,[5] strain H; and two strains of *Euglena gracilis* var. *bacillaris*, one green and the other colorless. The ciliate and the colorless *Euglena* were cultured in diffuse light; the green strain, under constant fluorescent illumination.

The counting procedure outlined by Hall, Johnson, and Loefer (1935) was used to determine the initial and final number of protozoan cells per milliliter medium in each culture. In order to facilitate comparisons between different series and species, the average cell number per milliliter medium in each group of test cultures at the conclusion of a period of incubation was expressed as a percentage of the average of the controls. Taking as a standard (100 per cent) the number of cells produced in the control cultures from the original inoculum, the percentage of cell numbers produced in the test cultures from the same amount of inoculum and in the same duration of time therefore represents the degree of change in cell numbers effected by the neomycin.

Size measurements were made with a calibrated ocular micrometer, using a 44× objective and a 10× ocular. The organisms were fixed with Bouin's solution and then immediately measured in their original culture medium in an open counting chamber.

[5] Dr. John O. Corliss (personal communication) designates *pyriformis* as the type species for *Tetrahymena*.

EXPERIMENTAL RESULTS

The purpose of the first series was to determine the effect of neomycin over a broad range of concentrations extending up to 2,000 units per milliliter at intervals of 400 units per milliliter. Aliquots of the same media were used for inoculation with *T. geleii* H and the colorless strain of *E. gracilis* var. *bacillaris*.

The initial count of the ciliate cultures averaged 2,300 cells per milliliter medium after inoculation. In the controls this number increased approximately sixty-eight times to attain a total of 157,400 per milliliter medium after 210 hours incubation. In those test cultures which contained the highest concentration of neomycin (2,000 units per milliliter), the number increased less than ten times, to a total of 22,000 per milliliter medium. The corresponding values for colorless *Euglena* were 350 cells at the start; this reached 213,600 in the controls, for an increase of 610 times. The number in the test cultures, however, merely doubled.

For comparison of the two species, the percentage of cell numbers in test cultures, relative to that in the controls, was 14 for the ciliates at 2,000 units per milliliter and 0.3 for the flagellates. This indicates that after 210 hours' incubation the inhibition of population growth was 86 per cent for *Tetrahymena* and 99.7 per cent for *Euglena;* at 1,200 units per milliliter, inhibition of the former was 48 per cent and that of the latter was 99.3 per cent. These findings, in addition to the results obtained at the other concentrations, are shown graphically in Figure 1.

Inhibition was evident even after a long period of time, as indicated by the counts made at 210 hours, and by viability tests after 7 weeks of incubation. The

control cultures were still viable, but there were no living organisms in any of the test cultures.

In the second series of experiments, the incubation period and the neomycin levels were reduced. Figure 2 illustrates the results obtained. The initial count per milliliter of medium was 1,150 for *Tetrahymena* and 380 for *Euglena*. After 72 hours' incubation, the number of the former increased to 83,000 in the controls, but was 142,000 in the cultures with 25 units per milliliter, or 71 per cent more cells than without neomycin. Stimulation was less evident as concentration of neomycin was increased, but the counts remained well above those of the controls, up to 250 units per milliliter. At 500 units per milliliter the tests were below the controls in number of cells. With *Euglena*, no significant difference was noted between the controls and the tests at the 25- and 50-unit levels, but inhibition was marked at higher concentrations.

It appeared from microscopic observation that the ciliates in the test cultures were much smaller than those in the controls. Measurements were made, and the averages of these, together with the standard deviations, were calculated. These data are given in Table 1. It is seen that after 72 hours in the 250-unit neomycin medium the cell length averaged only about half that of the control cultures, and the cell width was also considerably reduced.

A third series of experiments was performed to compare the green and colorless strains of *Euglena;* the results are shown in Table 2. There was apparently no significant difference in population growth inhibition of these strains.

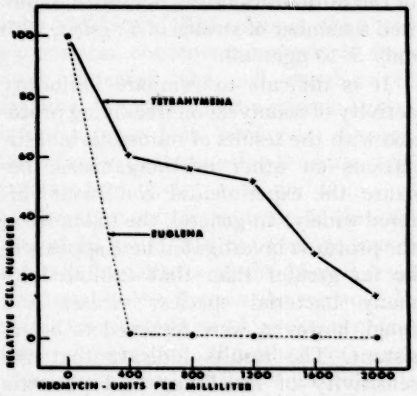

FIG. 1.—Effect of neomycin on Tetrahymena and Euglena at 210 hours.

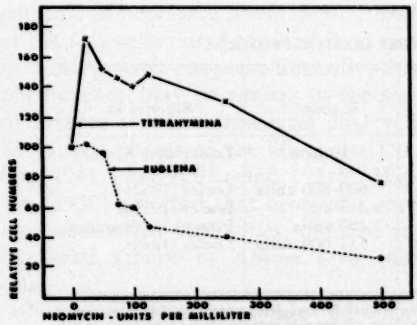

FIG. 2.—Effect of neomycin on Tetrahymena and Euglena at 72 hours.

TABLE 1

EFFECT OF NEOMYCIN ON SIZE OF *Tetrahymena*

Average dimensions in microns after 72 hours' incubation in various concentrations of neomycin.

Neomycin Units per Milliliter	Length	Width
0	71.2±2.88	33.5±1.31
25	58.0±3.54	28.2±1.72
50	46.5±3.50	25.8±1.21
75	46.0±2.62	27.7±1.85
100	39.4±1.78	26.9±1.13
125	39.8±2.08	28.0±1.43
250	34.1±1.22	26.7±0.99
500	38.3±1.21	22.9±0.91

DISCUSSION

Neomycin affected the population growth of the nonparasitic protozoa which were tested, and the flagellate was found to be more sensitive than the ciliate. Chlorophyll seemingly did not influence this response, as the green and colorless strains of *Euglena* were almost equally inhibited.

A tabulation was made of the activity of several antibiotics on the two species of protozoa which were investigated. The data in Table 3 were derived from numerous experiments in which the conditions—such as composition of medium, amount of inoculum, and temperature of incubation—were similar in all cases, with some relatively minor exceptions. The unit is not standard for the various antibiotics, and the weight basis also does not admit of direct comparison, as the potency per milligram often varies with the particular preparation which is used.

It may be noted in Table 3 that relatively large concentrations of antibiotic were required to block multiplication of these organisms, in comparison to the minute amounts which are often effective against sensitive bacteria. Only one of the antibiotics listed, actidione, inhibited a number of strains of *T. geleii*, with only 5–10 μgm/ml.

It is difficult to compare inhibitory activity of neomycin on free-living protozoa with the results of numerous investigations on other microörganisms, because the experimental conditions differed widely. In general, the tolerance of the protozoa investigated here appears to be far greater than that exhibited by many bacterial species; viruses and fungi, however, were reported to be resistant. The results indicate that the sensitivity of free-living and parasitic flagellates may be of the same order of magnitude.

TABLE 2

Euglena. COMPARISON OF GREEN AND COLORLESS STRAINS AFTER 120 HOURS IN CULTURES CONTAINING NEOMYCIN

Neomycin Units per Milliliter	No.*	Green Strain (Per Cent)	No.*	Colorless Strain (Per Cent)
0 (controls)	225.0	100	91.5	100
50	197.6	88	72.3	79
500	18.7	8	15.2	17
1,200	11.8	5	7.6	8
2,400	11.7	5	6.3	7

* Thousands of flagellates per milliliter medium.

TABLE 3

ANTIBIOTIC INHIBITION OF NONPARASITIC PROTOZOA*

Antibiotic	Incubation Time (Hr.)	*T. geleii*†	*E. gracilis*‡	Reported by
Actidione	48	5–10 μgm.	5–10 μgm.	Loefer and Matney (1951)
Bacitracin	144	>800 units	600–800 units	Loefer (1952)
Chloromycetin	144	1.8 mg.	>3.3 mg.	Loefer (1951*a*)
Neomycin	210	>2,800 units	400 units	Present investigation
Penicillin	96	10,000–25,000 units	133,000 units	Loefer (1949)

* Grown in Bacto Casitone media. Amounts of antibiotics required for complete or near-complete inhibition of population growth, based on counts and viability tests. Quantities expressed in concentration per milliliter of culture medium. μgm. = micrograms; mg. = milligrams; unit = standard potency for the particular antibiotic.

† Strain H; also others for actidione.

‡ Green and/or colorless strain.

The second series of experiments (cf. Fig. 2) demonstrated that proliferation of *T. geleii* H was stimulated at the lower concentrations of the neomycin preparation which was used, up to 72 hours of incubation. Inasmuch as the size of organisms in the neomycin-containing media was considerably smaller than in the control cultures, it appears that this effect was confined to an increase in cell number, and there may be no actual increase in the quantity of protoplasm. A similar effect has frequently been observed with bacteria in the case of penicillin (Pratt and Dufrenoy, 1949). Robbins, Hervey, and Stebbins (1950) found that the aureomycin concentrations which they used increased the cell numbers of *Euglena*. It appears that a mitotic stimulator is present in neomycin. Experiments are in progress using the pure components (neomycins A, B, and C).

Measurements were made of the ciliates at 72 hours, and the average size of the cells in cultures at the 25-unit level was notably less than in the controls. In media which contained higher concentrations of neomycin, the effect was even more pronounced. In this connection it is interesting to note that Weiss and Waksman (1950) observed marked changes in cell morphology of *E. coli* when it was cultured at neomycin levels slightly lower than those required for inhibition and in which growth was poor. Basically, this phenomenon may be similar to the size differences of microörganisms observed at various phases of the life-cycle (Dubos, 1944, chap. v; Loefer, 1951*b*). Wilson (1950) reported that neomycin, and also some other antibiotics, induced reductional groups in *Allium cepa* root cells.

No experiments were conducted to determine whether populations could be developed that would be tolerant to very high concentrations of neomycin. *Mycobacterium tuberculosis* H37RV, according to Steenken, Wolinsky, and Bolinger (1950), continued to grow in a medium containing 1,000 units per milliliter, and resistance was not lost after many transfers in media without neomycin. Weiss and Waksman (1950) noted a synergistic inhibitory effect when neomycin was tested in combination with streptomycin.

It was observed that a more concentrated culture broth and use of a heavier ciliate inoculum tended to counteract the inhibitory effect of neomycin. A similar correlation with amount of inoculum was noted for *E. coli* by Waksman, Katz, and Lechevalier (1950). As to the effect of other substances on potency of neomycin, 10 per cent beef serum had but little effect on test cultures of *M. tuberculosis* H37RV (Steenken, Wolinsky, and Bolinger, 1950). Waksman, Katz, and Lechevalier (1950) reported that activity was reduced only slightly by cysteine but considerably by oleic acid, nucleic acid, and glucose, whereas salt increased it.

SUMMARY

1. Changes in cell numbers of *T. geleii* H and *E. gracilis* var. *bacillaris* (green and colorless strains) when grown in Bacto Casitone–Bacto Yeast Extract medium with various concentrations of neomycin were investigated.

2. Proliferation of *Tetrahymena* was stimulated with 25–250 units per milliliter. Cell number in the medium containing 25 units per milliliter, after a 72-hour incubation period, exceeded that in controls by 71 per cent.

3. Ciliates grown without neomycin were larger than those from cultures containing the antibiotic; at 250 units per milliliter the difference in average length amounted to as much as 100 per cent.

4. There were fewer ciliates in cultures

with 400 or more units of neomycin per milliliter than in controls.

5. The colorless flagellate was more sensitive than *Tetrahymena*. Inhibition of population growth occurred at neomycin levels above 50 units per milliliter; when incubated for 210 hours in 400 units per milliliter, the inhibition was 99 per cent complete.

6. No significant difference was noted in the degree of inhibition exhibited on the green and colorless strains of *Euglena*.

7. The inhibitory effect of neomycin was compared with results reported for other antibiotics on these two species of free-living protozoa.

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THE MAGNESIUM REQUIREMENT OF TETRAHYMENA[1]

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INTRODUCTION

In a recent review, Ochoa (1951) has shown that most carboxylases require either Mg^{++} or Mn^{++} for activation. One of the major cellular sites of activity of magnesium is within the mitochondria. Hogeboom (1951) states that the mitochondria are chiefly concerned with the oxidative metabolism of the cell, including some of the reactions of the Krebs cycle. Lehninger shares this view (1951) and states that magnesium is essential for the activation of the enzymes of the succinoxidase system, which are virtually exclusively localized in the mitochondria.

The inorganic requirements of *Tetrahymena* in synthetic media have not been studied except for the papers from Kidder's laboratory (1949, 1951). The difficulties inherent in this kind of work are many. Traces of metals may be present as contaminants in many of the organic materials used, and the very metal tested may carry along traces of other metals. The glass equipment may also add extraneous metals to the test medium. All is not hopeless, however. If all growth is stopped when the metal in question is omitted and begins when the metal is added in very small quantities, it is improbable that other trace metals are significantly interfering. The possibility that trace metals in extremely small concentration may have an effect is, however, not absolutely ruled out.

Without quartz glassware, it is impossible to prevent the source of error from ordinary glass; but here again, if no growth is obtained in the usual Pyrex ware, the influx of other ions must be slight. Purification of the organic materials is beyond the resources of the usual physiological laboratory, but, once more, lack of growth in the absence of the test metal makes it unlikely that significant contaminants are present.

A complete purification of all the ingredients used in the diet of *Tetrahymena* is a major and a very difficult task at this time. The minimal magnesium requirement of this protozoan is not established by this work, but the amount of analytic-grade magnesium sulphate necessary for full growth has been determined.

MATERIAL AND METHODS

The organism used in this investigation was *Tetrahymena geleii* (strain E). Stocks were grown in a mixture of proteose peptone and bacto-tryptone (Table 1). The pH of this stock medium was adjusted to 7.4 with NaOH. Cultures were kept in this medium for 48 hours and then separated from the medium by means of centrifugation with an International Clinical centrifuge. The centrifuging time was 45 seconds at 1,200 r.p.m. (100 × g). After the final wash, the animals were suspended in double-distilled water and adjusted by either further centrifugation or dilution to a concentration

[1] From a dissertation presented to the Horace H. Rackham School of Graduate Studies in the University of Michigan, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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which would provide approximately one thousand animals per tube upon inoculation.

All tests and controls were undertaken in duplicate tubes containing 5 ml. of synthetic medium, as recommended by Elliott (1949*a*, 1950) (Table 2). However, biotin and choline were omitted, and the nucleic acid level was set at 200 γ/ml. Prior to inoculation, all media were sterilized by autoclaving at 15 pounds pressure and 118°–121° C. for 10 minutes. The salts and vitamins were prepared as concentrates in order to reduce the error in weighing. The amino acids were supplied by Merck and Company, Inc., and all other chemicals were of analytic grade as supplied by Baker and Adams. Dr. E. L. R. Stokstad, of the Lederle Laboratories Division of the American Cyanamid Company, kindly supplied the protogen. Analytic-grade magnesium sulphate was used as the magnesium source, the metallic impurities here amounted to about 0.05 per cent.

Cultures were incubated at 25° ± 1° C. in the dark and in an upright position. The hydrogen-ion concentrations were adjusted by means of either NaOH or HCl to pH 6.5 or 7.5, depending on the experiment. The Beckman pH Meter, Model G, was used for checking this.

Aseptic technique was employed throughout this series of investigations, and water which was double-distilled in Pyrex was used in the preparation of all media.

Representative initial inocula were stained with freshly made 1:1 Bouin-stock eosin mixture. After a few minutes, 0.1-ml. volumes of this mixture were smeared on glass slides and allowed to dry. Counts were made by means of microscopic examination. The 10× wide-field ocular and the 10× objective were found to be best suited for counting the cells, and a mechanical stage facilitated scanning of the slide so as to include all areas of the smear. The eosin made the animals easy to find.

TABLE 1

MEDIUM USED FOR THE GROWTH OF STOCKS OF *Tetrahymena geleii* E

	Gm.
Proteose-peptone	5
Bacto-tryptone	5
$NaC_2H_3O_2 \cdot 3H_2O$	1
Glucose	1
KH_2PO_4	1
Yeast extract	0.1
Thiamin HCl	0.002
Distilled water	To 1 liter

TABLE 2

BASAL MEDIUM USED IN GROWTH STUDIES OF *Tetrahymena geleii* E

Substance	γ/Ml
l-Arginine monohydrochloride	600
l-Histidinemonohydrochloride	150
dl-Isoleucine	75
dl-Leucine	75
l-Lysine monohydrochloride	150
dl-Methionine	150
dl-Phenylalanine	150
dl-Serine	300
dl-Threonine	300
dl-Tryptophan	150
dl-Valine	150
Sodium acetate	1,000
Glucose	1,000
Calcium pantothenate	0.10
Nicotinamid	0.10
Pyridoxine hydrochloride	2.00
Riboflavin	0.10
Folic acid	0.01
Thiamin hydrochloride	1.00
Yeast nucleic acid	200.00
KH_2PO_4	100.00
$CaCl_2 \cdot 2H_2O$	50.00
$FeCl_3 \cdot 6H_2O$	1.00
$FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$	5.00
$CuCl_2 \cdot 2H_2O$	5.00
$MnCl_2 \cdot 4H_2O$	0.05
$ZnCl_2$	0.05
Protogen	1 unit

An increase in protoplasmic mass was interpreted as growth. This was determined turbidimetrically by means of the Klett-Summerson photoelectric colorimeter, in which the blue filter (420 mμ) was used as recommended by Elliott (1949*b*). The results are the averages from two or more experiments.

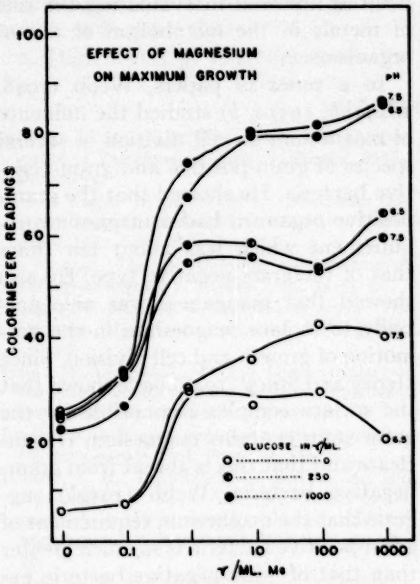

FIG. 1.—The effect of magnesium on maximal growth.

Since magnesium plays an important role as a coenzyme in the glycolytic cycle, it was decided to evaluate the effects of magnesium in respect to the amount of glucose substrate. Glucose was therefore varied from 0 to 0.1 per cent. Magnesium was tested in concentrations which ranged from 0 to 1,000 γ/ml.

RESULTS

In media containing no glucose at pH 6.5, maximal growth was obtained upon the addition of 1 γ/ml of magnesium (Fig. 1). Better growth was obtained at pH 7.5 when the level of magnesium was raised to 100 γ/ml (Fig. 1). The highest concentration of magnesium (1,000 γ/ml) resulted in about one-third growth inhibition, but only at the lower pH.

When the glucose level was raised to 250 γ/ml, it was found that as little as 0.1 γ/ml of the metal produced a definite growth response. Maximal growth for this level of glucose was obtained upon the addition of 1 γ/ml of magnesium.

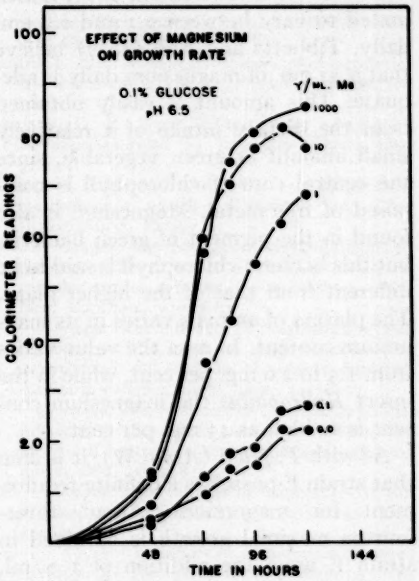

FIG. 2.—The effect of magnesium on growth rate

With glucose present, slight growth increases were obtained when magnesium was present to the extent of 0.1 γ/ml. More than three-quarters maximal growth was obtained by the addition of 1 γ/ml of magnesium in media containing the highest amount of glucose.

Full growth was obtained with 10 γ/ml of magnesium, and higher levels were without effect in media containing 0.1 per cent glucose.

Both maximal growth (Fig. 1) and the growth rate (Fig. 2) were affected by the

amount of magnesium present up to 10 γ/ml. Much slower growth rates were obtained in magnesium-deficient media. Above the 10 γ level of magnesium, little change was obtained in maximum growth with glucose present.

DISCUSSION

Magnesium is known to be essential for life. The requirement for magnesium is not known in the human, but it is estimated to vary between 0.2 and 0.6 gm. daily. Tibbetts and Aub (1937) believe that 0.22 gm. of magnesium daily is adequate. This amount is easily obtained from the dietary intake of a relatively small amount of green vegetable, since the central core of chlorophyll is composed of this metal. Magnesium is also found in the pigment of green bacteria, but this bacterio-chlorophyll is said to be different from that of the higher plant. The plasma of animals varies in its magnesium content. In man the value varies from 1.5 to 2.6 mg. per cent, while in the insect *Hydrophilus* the magnesium content is as high as 43 mg. per cent.

As with *T. geleii* (strain W), it is seen that strain E possesses a definite requirement for magnesium. About three-fourths maximal growth is obtained in strain E upon the addition of 1 γ/ml, which compares favorably with Kidder's findings (1951), where 2 γ/ml were needed for maximum growth in strain W.

Although the analytic grade of magnesium sulphate used had a total of 0.05 per cent impurity, it is improbable, though not impossible, that the growth obtained upon the addition of 0.1 γ/ml of the salt was due to trace substances. The total contamination from this source would be of the order of 0.005 γ/ml. This is of a smaller magnitude than of any of the growth factors needed.

Protogen, with its unknown composition, obscures the inorganic picture. From the method of preparation of this material, however, metals may not be present to any great extent.

Hutner *et al.* (1950) in an interesting paper have pointed out the technical difficulties involved in evaluating the role of metals in the metabolism of microorganisms.

In a series of papers, Webb (1948, 1949*a*, *b*, 1951*a*, *b*) studied the influence of magnesium on cell division in several species of gram-positive and gram-negative bacteria. He showed that the gram-positive organism had a magnesium requirement which was about ten times that of the gram-negative type. He also showed that manganese was able partially to replace magnesium in the promotion of growth and cell division. Since Henry and Stacy (1946) have shown that the surface complex responsible for the gram stain contains magnesium ribonucleate and that this is absent from gram-negative bacteria, Webb (1949*b*) suggests that the magnesium requirement of gram-positive bacteria is so much greater than that of gram-negative bacteria because of the incorporation of the metal into the structure of the gram complex. It was also shown by Webb (1948) that cell division in *Clostridium* was inhibited in magnesium-deficient medium and that filamentous cells were produced instead of the customary rods.

It would be of interest to study the effect of magnesium deficiency on the morphology of *Tetrahymena*.

Elliott and Kalnitsky (1950) showed that magnesium ion was essential for the oxidation of acetate by the rabbit kidney cortex. Seaman (1950) demonstrated that acetate was utilized by his strain of *Tetrahymena* and that 14 per cent of the acetate was oxidized. These experiments were undertaken in Hahnert's solution,

which contains magnesium as one of the components. It is not known, however, whether magnesium is essential for the oxidation of acetate in this invertebrate.

SUMMARY

1. Magnesium is essential for full growth of *T. geleii* E.

2. High levels of magnesium are inhibitory in the absence of glucose in media on the acid side of neutrality.

3. A definite growth increase is obtained upon the addition of as little as 0.1 γ/ml of magnesium in the presence of glucose.

4. Maximum growth is obtained by the addition of 10 γ/ml of magnesium in media containing 0.1 per cent glucose.

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GROWTH OF CHILOMONAS PARAMECIUM IN SIMPLE ORGANIC MEDIA

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ALTHOUGH there are some data on the ability of *Chilomonas paramecium* to grow on various organic compounds (Mast and Pace, 1933; Hutchens, 1948; Pace, 1948; Cosgrove, 1950), no systematic study of growth on a series of homologous compounds in

[1] The material on the monohydric alcohols was submitted by the junior author as a thesis for the degree of Master of Science.

media of known composition has been available. This present work was undertaken to determine whether, in the absence of other appreciable organic sources of carbon, the ability of *C. paramecium* to utilize aliphatic alcohols, organic acids, and sugars showed any systematic relationship to the structures of the compounds. Interest was centered on the ability of organisms equipped with the enzyme systems permitting growth in ethyl alcohol medium to utilize other organic compounds upon their first exposure to them. No attempt was made to adapt the organisms to new carbon sources[2] by prolonged cultivation in their presence.

MATERIALS AND METHODS

Preparation and sterilization of glassware and media followed the procedures of Cosgrove (1950). The basal salt solution was the same as that used by Hutchens (1948). All chemicals used were ACS or Eastman grade.

Stock solutions of the alcohols were prepared by adding the required amount of alcohol to 50 ml. of sterile basal medium; stock solutions of acids, by adding the required amount of concentrated acid to 50 ml. of basal medium, adjusting to pH 6.0–6.1 with N/1 NaOH, and autoclaving. Aliquots of the stock solutions were then added aseptically to sterile basal medium in culture tubes to give the final concentration desired.

Stock cultures of the Pringsheim strain of *C. paramecium* obtained from Professor R. P. Hall were maintained in the basal medium with ethyl alcohol as a carbon source. Inocula for experimental cultures were taken from stocks in the maximum stationary phase; the ratio of inoculum to final culture volume was always 1:100.

Experimental cultures were grown in Pyrex test tubes covered with aluminum caps and incubated at 25° C. in a slanted position. At intervals the optical density of the culture was determined by Elliott's (1950) technique in a Coleman spectrophotometer at 400 mμ. When three consecutive similar optical-density readings indicated that the maximum population had been attained, an aliquot was removed from each culture for sterility tests. One-tenth volume of Bouin's fluid was added to the remainder, and population counts were made. In the studies involving growth curves, no counts were made after it became apparent that there was excellent agreement between optical-density readings and population through the maximum stationary phase. Careful attention to the volume of inoculum and culture medium was found to be necessary to obtain good agreement among similar tubes.

For each compound tested, three sets of tubes were used. Set 1 contained no added carbon source, and the slight growth in these tubes resulted from substances carried over with the inoculum or introduced with the salts of the basal medium. Set 2 contained the compound to be tested; set 3 contained both the compound to be tested and ethyl alcohol or acetate and served to reveal any toxic effects of the test substance. In addition, tubes containing ethyl alcohol or acetate were inoculated as controls for the suitability of the culture medium and viability of the inoculum. If the maximum population in set 3 did not equal or exceed that in the control tubes, the experiment was repeated with lower concentrations of the test compound. By this method, a single transfer furnished infor-

[2] "Carbon source" signifies an organic compound serving as a source of both carbon and energy.

mation available only after several transfers in the usual serial transfer technique.

RESULTS

The results are summarized in Figure 1 as the ratio of the average maximum population in tubes of set 2 or of set 3 (N_x) to the average maximum population in tubes of set 1 (N_B). It is apparent that only the even-number, straight-chain acids and monovalent alcohols support growth comparable with that in ethyl alcohol or acetate. Branched-chain, C_1, and polyvalent compounds are not utilizable carbon sources, and they do not interfere with growth on ethyl alcohol or acetate. The straight-chain, odd-number compounds above C_1, at concentrations which are not lethal for their isomers, interfere with growth on utilizable carbon sources. Microscopic examination of these cultures showed that the inoculum died out over a period of several days, whereas the lowest lethal concentration of any of the compounds killed off the inoculum within 24 hours.

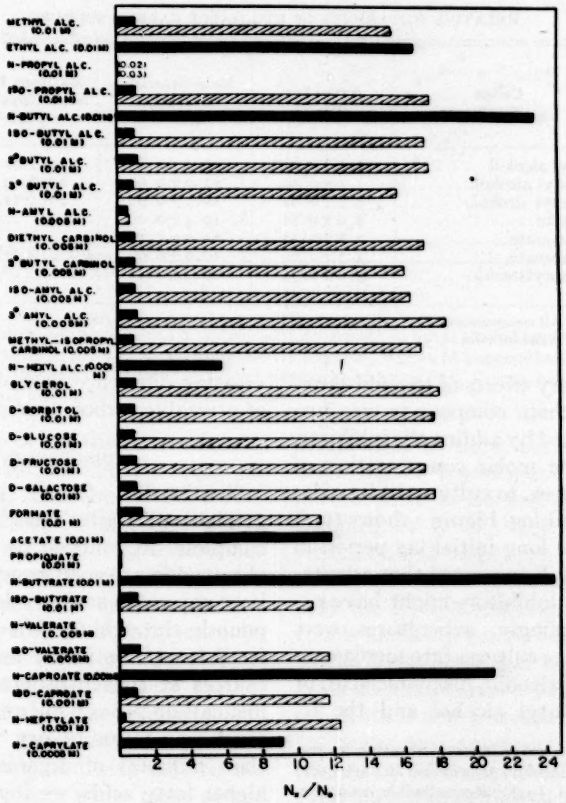

FIG. 1.—Growth on various carbon sources. *Solid bars*, indicated compound alone; *shaded bars*, indicated compound plus 0.01 M ethyl alcohol (alcohols) or 0.01 M acetate (acids).

The relative suitability of the utilizable compounds was determined by measuring initial lag, mean generation time, and, for the alcohols,[3] maximum population for each compound in cultures inoculated and incubated simultaneously. Table 1 shows that, so far as the criteria are indicative, all seven compounds are equally satisfactory. When large numbers of organisms are desired for experimental purposes, *n*-butyl alcohol at 0.01 M is the best carbon source, giving populations of about 1,000,000/ml.

TABLE 1

RELATIVE SUITABILITY OF UTILIZABLE CARBON SOURCES

Carbon Source	Initial Lag (Hours)	Mean Generation Time (Hours)	Maximum Population (Organisms per Mm^3)
Ethyl alcohol	3.0±0.25†	10.2±0.83	245±20.7
n-Butyl alcohol*	3.5±0.43	11.0±0.66	259± 9.1
n-Hexyl alcohol	4.1±0.62	10.7±0.92	261±12.6
Acetate	2.9±0.81	10.5±0.29	.....
n-Butyrate	3.2±0.33	11.9±1.76	.....
n-Caproate	3.7±0.60	10.8±0.06	.....
n-Caprylate	3.8±0.59	10.3±0.45	.....

* All compounds used at concentrations supplying 72 μgm carbon/ml.
† Standard error.

The inhibitory effects of the odd-number, straight-chain compounds was further investigated by adding the inhibitor, at one-half the molar concentration of the carbon source, to cultures at inoculation or near mid-log. Figure 2 shows typical results; the long initial lag period in cultures *D* and *E* suggested that adaptation to the C_3 inhibitors might have occurred. Accordingly, subcultures were made from these cultures into media containing propyl alcohol, propionic acid, or mixtures of butyl alcohol and the C_3 compounds. No growth occurred with the C_3 compounds alone, and the long initial lag period reappeared in the medium containing *n*-butyl alcohol and C_3 compounds. The growth finally obtained in cultures *D* and *E* does not indicate any permanent adaptation to the inhibitors.

The results with various combinations of odd-number and even-number straight-chain compounds showed that the homologous inhibitors were always more effective than the nonhomologous and that effectiveness decreased with increasing difference in molecular weights of utilizable carbon source and inhibitor.

[3] Increase of pH, not exhaustion of carbon source, determines the maximum population in fatty acid cultures (Hutchens, 1948). Reproducible maximum populations could not be obtained when the alkali accumulating during growth was periodically neutralized with HCl.

DISCUSSION

Reinhardt's (1951) paper became available only after this study was nearly complete. In contrast to our results, he obtained fair to good growth on the lower iso-compounds and on odd-number compounds through C_5. His technique differed from ours in the use of all carbon sources at concentrations supplying 50 mg. carbon per 100 ml. and in expressing results as ratios of dry weights rather than numbers of organisms. With the higher fatty acids, we found that molar concentrations corresponding to his carbon levels were lethal. His basal medium

was similar to ours except for the addition of trace elements, which we have considered to be supplied in adequate amounts by impurities in our salts. It is possible that his strain differs from ours in the profound ways suggested by the difference in results. There are no published data on strain differences in *Chilomonas*, but our inability to reduce mean generation time to less than *ca.* 10 hours, as compared with Hutchens' (1948) 5.6 hours, indicates that such differences may exist. At present, it seems impossible to account for the lack of agreement between our work and Reinhardt's.

Provasoli's (1938) experiments are not strictly comparable with ours, since he used a salt-peptone basal medium. However, in general agreement with our results, he found that the even-number, straight-chain compounds supported good growth compared with that on the basal medium alone, while most of the other compounds tested did not support any better growth than the basal medium alone. He did not report any inhibition with odd-number straight-chain compounds.

The parallelism between the types of alcohols and acids utilized is to be expected if the first step in the utilization of the alcohol is its oxidation to the corresponding acid (Blum, Podolsky, and Hutchens, 1951). The failure of the C_1 compounds to show the same effect as their higher homologues is to be expected, since, as the first members of the homologous series, they possess somewhat atypical properties.

The ability of *Chilomonas* to utilize only the straight-chain even-number alcohols and acids tested offers no obstacle to the hypothesis that the larger molecules are first converted to C_2 fragments (cf. Hutner and Provasoli, 1951, p. 76). In theory, this conversion can be accomplished by successive β-oxidations. However, such β-oxidation, if it occurs, must be different from that in higher forms, where it can take place with any fatty

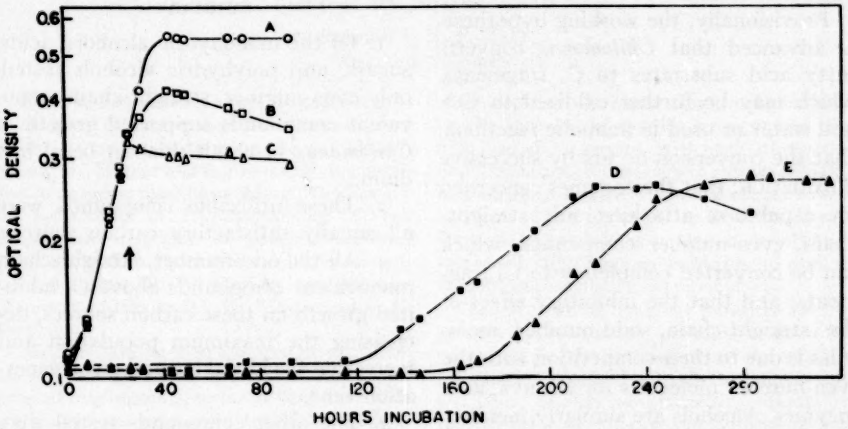

FIG. 2.—Effect of propyl alcohol and propionic acid on growth in butyl alcohol medium. Basal medium plus 0.005 M butyl alcohol; pH 6.1; 25° C. Additions: *A* (control), 0.0025 M isopropyl alcohol; *B*, 0.0025 M propionic acid at 26 hours (↑); *C*, 0.0025 M propyl alcohol at 26 hours (↑); *D*, 0.0025 M propionic acid at inoculation; *E*, 0.0025 M propyl alcohol at inoculation.

acid. If this same type occurred in *Chilomonas*, the C_2 fragments accumulating from the oxidation of the higher iso-compounds should permit detectable growth on these compounds. Absence of growth indicates that the compounds are not attacked. It is also difficult to account for the inhibitory action of the odd-number, straight-chain compounds if typical β-oxidation occurs.

Provisionally, the working hypothesis is advanced that *Chilomonas* converts fatty acid substrates to C_2 fragments, which may be further oxidized to CO_2 and water or used in anabolic reactions; that the conversion occurs by successive β-oxidation; that the enzymes concerned are capable of attacking only straight-chain, even-number compounds, which can be converted completely to C_2 fragments; and that the inhibitory effect of the straight-chain, odd-number molecules is due to their competition with the even-number molecules for the available enzymes. Alcohols are similarly metabolized after oxidation to the corresponding fatty acid.

The present study confirms the inability of *Chilomonas* to utilize sugars, indicating that the synthesis of reserve carbohydrate must proceed by some pathway other than that found in higher forms. The inability to utilize glycerol raises several questions which cannot at the present be answered concerning the synthesis and utilization of the reserve fats found in the organisms.

SUMMARY

1. Of the monohydric alcohols, acids, sugars, and polyhydric alcohols tested, only even-number, straight-chain monovalent compounds supported growth of *Chilomonas* in a salt-thiamin basal medium.

2. These utilizable compounds were all equally satisfactory carbon sources.

3. All the odd-number, straight-chain monovalent compounds above C_1 inhibited growth on these carbon sources, decreasing the maximum population and increasing the initial lag and mean generation time.

4. The other compounds tested were without effect.

5. A working hypothesis based on these results is suggested.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

BIOCHEMISTRY AND PHYSIOLOGY OF PROTOZOA, VOLUME I[1] EDITED BY ANDRÉ LWOFF

Reviewed by R. P. HALL
New York University

This volume, which is unique in modern protozoölogical literature, includes an introduction by the editor and sections on the phytoflagellates (S. H. Hutner and L. Provasoli), the nutrition of parasitic flagellates (Marguerite Lwoff), the metabolism of Trypanosomidae (T. von Brand), the nutrition of parasitic amoebae (Marguerite Lwoff), the biochemistry of malarial parasites (R. W. McKee), and the biochemistry of ciliates (G. W. Kidder and V. C. Dewey).

Modern data on food requirements and metabolism are discussed in detail. Nitrogen and mineral requirements, so far as they have been established, are summarized for phytoflagellates and ciliates, the only groups for which fairly extensive data have been accumulated. Available information on the metabolism of carbon compounds is presented for the phytoflagellates, Trypanosomidae, malarial parasites, and ciliates. The role of acetate as a substrate for many phytoflagellates is stressed, with particular reference to the status of a tricarboxylic acid cycle in these organisms. Vitamin requirements are listed for the phytoflagellates and *Tetrahymena*, and certain apparent requirements of malarial parasites, trypanosomes, Endamoebidae, and trichomonad flagellates are discussed. Among the more recent developments reviewed are the vitamin B_{12} requirement of *Euglena gracilis* (and its applicability to bio-assays), protogen as an essential growth factor for *Tetrahymena*, the apparent requirement of linoleic acid by *Trichomonas vaginalis*, and that of cholesterol by *Entamoeba histolytica*.

Studies on oxygen consumption and oxidative mechanisms in trypanosomes are reviewed, with accent on the interesting problems raised by the existence of cyanide-sensitive and cyanide-insensitive species. With such information as a foundation, the activity of certain trypanocidal drugs is related to specific effects on metabolism of the parasites. In addition, the effects of trypanosomes on the metabolism of their hosts and the possible bearing of such effects on pathogenicity are pointed out.

The section on malarial parasites reviews modern progress in the attempts to grow the organisms *in vitro* and points out the valuable information obtained with the techniques now available. Studies on glycolysis, vitamin requirements, and oxidative mechanisms have already made it possible to trace effects of certain chemotherapeutic agents to actions on specific enzymes, with obvious potential benefits in the search for better malaricidal drugs.

Perhaps the most noteworthy feature of the chapter on ciliates is the review of amino acid requirements in *Tetrahymena*. The accumulation of such information is an important milestone in protozoan physiology, since this ciliate, as the first heterotrophic protozoon for which such data have become available, will serve as a standard of comparison in subsequent studies on nitrogen requirements of the higher Protozoa. However, the determination of nitrogen requirements has been only one aspect of the problem in the development of chemically defined media for a ciliate. Identification of essential vitamins has reached much the same state of exactness, and, with the exception of protogen, the food requirements of *Tetrahymena* now seem to be established precisely, in a chemical sense. Perhaps the recent observation that lipoic acid can replace protogen will remove the

[1] New York: Academic Press, Inc., 1951. Pp. x + 434. $8.80.

one remaining chemical ambiguity. The chapter on ciliates is concluded with a discussion of *Tetrahymena* as an organism suitable for various microbiological assays. A list of media for such assays is appended.

Considered collectively, the various chapters present a broad review of protozoan food requirements and metabolic pathways, as well as fundamental problems in the chemotherapy of protozoan infections. Furthermore, the various authors have taken pains to point out questions which are still unanswered and to indicate paths of investigation which remain essentially unexplored. On either basis, the volume will be a valuable addition to the libraries of all who are interested in the study of Protozoa.

ENZYMES AND ENZYME SYSTEMS,[1] EDITED BY J. T. EDSALL

Reviewed by MAURICE SUSSMAN
Northwestern University

THIS volume is the first in an anticipated series of publications entitled "Memoirs of the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University." It is not, nor is it intended to be, a comprehensive treatment of enzymes or of enzyme systems. It is, rather, an arbitrary compilation of writings describing some recent fundamental contributions to the discipline of enzymology. The authors were among the participants in a symposium on the state in nature of proteins and protein-enzymes which was held at Harvard.

A. L. Lehninger has contributed a chapter on the respiratory activity of isolated rat-liver mitochondria. The results of a number of investigations have been integrated and indicate convincingly that the mitochondrial fraction contains a bound, organized system of enzymes capable of mediating the complete oxidation of fatty acids via the Krebs cycle and cytochrome system and of accomplishing the concomitant esterification of phosphate.

A review of the work on the cyclophorase system has been written by D. E. Green. The studies beautifully complement the material in the first chapter. Details are given of the preparation of the cyclophorase gel and its enzymic capabilities. Cofactor and primer requirements are mentioned. The ability of the system for oxidative phosphorylation is described.

E. L. Smith has presented a particularly elegant discussion of the specificity of peptidases. The nature of the primary and secondary valence binding between substrates and the metal-activated enzymes is elucidated with the aid of information concerning the comparative abilities of various synthetic peptides to combine both with the metal-enzyme complexes and with free metal ions.

A description is given by A. C. Maehly of the reactions undergone by the apoenzyme of horse-radish peroxidase with various hemins, including the normal prosthetic group of this enzyme. B. Chance provides a discussion of the reaction kinetics of the peroxidase-ferrocytochrome C redox system. He demonstrates that the velocity of the reaction is too great to be accounted for by simple iron-iron collisions between the reactants. It is postulated that peripheral collisions between the enzyme-proteins can effect electron transfer.

Last, a chapter by E. J. Cohn, D. M. Surgenor, and M. J. Hunter is devoted to the "State in Nature of Proteins and Protein Enzymes of Blood and Liver." It contains a compendium of methods devised primarily by the Harvard group for the gentle extraction and separation of protein tissue components with a minimum alteration of biological specificity.

[1] Cambridge, Mass.: Harvard University Press, 1951. Pp. 142. $2.50.

COMPARATIVE PHYSIOLOGY OF THE THYROID AND PARATHYROID GLANDS[1] BY WALTER FLEISCHMANN

Reviewed by DIETRICH C. SMITH
University of Maryland

THIS is a short, compact, but somewhat incomplete account of the comparative physiology of the thyroid and the parathyroid glands, useful to the general student. The some two-hundred-odd papers listed in the Bibliography are obviously selected, since the list is far from exhaustive. The book reflects the rising interest in the subject of comparative endocrinology and its growing bounds; it is the fourth review of the comparative physiology of the thyroid to appear in one form or another during the past few years.

Admittedly, we know little about the physiology of the thyroid in the fishes, but what we do know is considerably more extensive than is reported here. This is perhaps an unfortunate weakness, since the fishes, because of their phylogenetic position, are a particularly interesting group and deserve more attention than they have received in the past. There is no discussion of the growing tendency to associate the thyroid with salt metabolism and osmoregulation in the fish or of the fact that the scanty evidence available seems to implicate the thyroid in adaptations to unfavorable environmental situations.

The discussion of the function of the thyroid in amphibians is far more complete, and the summary of the literature dealing with the effect of the thyroid on metamorphosis is excellent. As in the fish, there is very little evidence that the thyroid in any way controls oxygen consumption, but here the resemblance ends; for in the amphibian, contrary to the fish, there is every evidence that the thyroid is intimately concerned in the processes of growth and differentiation, particularly the latter.

There has been surprisingly little work done on the function of the thyroid in the reptiles and birds. It is the virtue of works of this sort that attention is directed to such glaring gaps in our knowledge. The little investigation that has been made on the reptiles seems inconclusive and deals primarily with the effect of the thyroid on molting, while that done on birds is concerned mainly with the effect of the thyroid on plumage. Apparently no one has thought of making a serious study of the metabolic effects of the thyroid in the reptiles. This is an unfortunate omission, since this group stands on the threshold of a warm-blooded existence, and it is in the warm-blooded forms that the thyroid has assumed its dominant role in facilitating organismal adaptations to changes in the temperature of the external environment.

The most extensive discussion concerns mammals, and over half of the book is devoted to this group. Consideration is given to the effect of the thyroid hormone in the monkey, dog, rabbit, calf, rat, goat, sheep, and pig, and attention is thus focused on numerous species differences. As is quite proper in a book of this kind, there is little concerning its action in man, and clinical matters are entirely omitted. Most of the discussion centers around the relation of the thyroid gland to ossification and bone development, growth, age, cholesterol metabolism, iodine metabolism, lactation, and temperature regulation. Surprisingly there is only a bare mention of the relation of the thyroid to reproductive activity and very little on the mechanisms regulating thyroid activity.

The discussion of the physiology of the parathyroid gland is excellent, and the material presented is quite inclusive. It is interesting to note that nothing is known of the parathyroids in the fish and apparently in the reptiles as well. In the amphibians, birds, and mammals, where removal of the parathyroids has been attempted, the result is uniformly a fall in blood calcium.

[1] Springfield, Ill.: Charles C. Thomas, Publisher, 1951. Pp. 78. $2.25.

THE INTEGUMENT OF ARTHROPODS,[1] BY A. GLENN RICHARDS

Reviewed by CARROLL M. WILLIAMS
Harvard University

THE skin of an insect is simultaneously a skeleton, an armor, a camouflage, and a principal route of exchange between the inclosed life and the external environment. A "skin-deep" understanding of an insect can therefore be a substantial understanding of that insect. Moreover, the nature of insects is such that an increasing number of scientific workers find practical concern in the properties of this physical-chemical-biological system, the insect skin.

A diverse scientific audience will therefore rejoice in the publication of *The Integument of Arthropods*—the long-awaited monograph by Professor A. Glenn Richards, of the University of Minnesota.

As the foremost American authority in this field, Richards considers the organization and properties of the arthropod integument in physical, chemical, and biological terms. The difficulties of this task can scarcely be exaggerated. Few, for example, would suppose that over nineteen hundred scientific papers have been published pertaining more or less directly to the insect cuticle. Yet Richards seems intimately familiar with all of them and finds his way through the jungle with a facility to be compared only with that of Joseph Needham. In doing so, it is fortunate that the author does not withhold his judgment concerning the good and bad in this diverse and frequently contradictory literature. Where more work is needed, this, too, is stated in no uncertain terms.

The monograph is divided into three sections: fourteen chapters descriptive of the chemical and biochemical organization, thirteen chapters pertaining to the micro-anatomy and development, and three chapters concerned with the permeability to water, gases, simple chemicals, and insecticides. Fourteen tables summarize the literature, and some two hundred line drawings, diagrams, and photomicrographs record chemical configurations and structural properties. The author and publisher deserve special congratulations for citing the full titles of each reference in the bibliographic index, along with page notations to the text. The monograph terminates with a detailed subject index.

This first and only comprehensive account of the arthropod integument is, therefore, a mature analysis of the matter. The book can confidently be recommended to investigators in fields pertaining to the chemistry and physiology of insects. To investigators and teachers of entomology it is, of course, a "must."

[1] Minneapolis: University of Minnesota Press, 1951. Pp. 411.

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